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TITLE: PRODUCTION OF ENZYMATICALLY ACTIVE HUMAN ACETYLCHOLINESTERASE IN E. COLI

PRINCIPAL INVESTIGATOR: Marian Gorecki, Ph.D.

CONTRACTING ORGANIZATION: Bio-Technology General Corporation
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Human acetylcholinesterase (hAChE) was expressed in *Escherichia coli* under regulation of the constitutive deo promoter or the thermoinducible $\lambda P_L$ promoter. To facilitate the expression in the prokaryotic system, the recombinant human AChE (rhAChE) cDNA was modified at the N-terminus, by site-directed mutagenesis, in order to replace some of the guanisine and cytosine (GC)-rich regions by adenine thimidine (AT). These modifications did not alter the amino acid sequence but resulted in ample production of the protein. rhAChE accumulated in the cells and reached a level of 10% of total bacterial proteins. A partially purified inactive recombinant protein was recovered from inclusion bodies. Active rhAChE was obtained after solubilization, folding and oxidation; however, the overall yield of the active enzyme was low. A 20- to 40-fold increase in the process yield of active rhAChE activity was achieved by replacing $Cys^{580}$ by Ser. Substrate specificity and inhibitor selectivity of the recombinant mutant enzyme harboring Ser at amino acid number 580 were indistinguishable from those of the native AChE isolated from human erythrocytes.

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4. **Principal Investigators:** **5. Telephone No.: 972-8-381223**

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6. **Institution: Bio-Technology General**

7. **Project Title: Production of enzymatically active human AChE in *E. coli***

8. **Staff, with percent effort of each on project:**

| | | |
|---|---|---|
| **M. Fischer, Ph.D.** | **-** | **80%** |
| **G. Efrony, Res. Ass.** | **-** | **100%** |

9. **Contract expenditure:**

| | |
|---|---|
| **Personnel** | **199,913** |
| **Travel** | **-** |
| **Supplies** | **42,141** |
| **Over Head** | **212,751** |
| **Fee** | **26,158** |
| **Total** | **480,963** |

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# SUMMARY

Human acetylcholinesterase (hAChE) was expressed in *Escherichia coli* under regulation of the constitutive deo promoter or the thermoinducible $\lambda P_L$ promoter. To facilitate the expression in the prokaryotic system, the recombinant human AChE (rhAChE) cDNA was modified at the N-terminus, by site-directed mutagenesis, in order to replace some of the guanisine and cytosine (GC)-rich regions by adenine thimidine (AT). These modifications did not alter the amino acid sequence but resulted in ample production of the protein. rhAChE accumulated in the cells and reached a level of 10% of total bacterial proteins. A partially purified inactive recombinant protein was recovered from inclusion bodies. Active rhAChE was obtained after solubilization, folding and oxidation; however, the overall yield of the active enzyme was low. A 20- to 40-fold increase in the process yield of active rhAChE activity was achieved by replacing $Cys^{580}$ by Ser. Substrate specificity and inhibitor selectivity of the recombinant mutant enzyme harboring Ser at amino acid number 580 were indistinguishable from those of the native AChE isolated from human erythrocytes.

Scale-up of fermentation to 750L was performed with the clone expressing the rhAChE mutant, harboring a $Cys^{580} \rightarrow Ser^{580}$ substitution. The fermentation yielded a large amount of the inactive protein that was refolded *in vitro* on a pilot scale. Although the yield of refolding was low (~1%), about 500 mg of active rhAChE (specific activity 4572-5900 U/mg protein) was purified by DEAE-Sepharose and affinity chromatography to apparent homogeneity. The purified enzyme displayed substrate specificity and selective inhibition by BW284C51 and iso-OMPA, indistinguishable from those of the naturally occurring human erythrocyte-derived enzyme. The purified rhAChE has an ultraviolet absorbance spectrum typical of a tryptophan (Trp)-rich protein with a distinct shoulder at 290 nm, and a high absorption coefficient at 280 nm, $\varepsilon_{1\%}$ = 23.1. The Trp residues in active rhAChE are located in an apolar environment, characteristic of a globular molecule. The difference in amino acid composition between the erythrocyte-derived and recombinant hAChE is reflected in their different pI values: 5.5 - 5.8 and 4.6 - 5.2 for the recombinant and erythrocyte enzymes, respectively. The circular dichroism (CD) spectrum of rhAChE is typical for an α/ß protein: 39% α helix, 22% ß-sheet. This secondary structure is similar to that determined for the *Torpedo* AChE, by both CD and X-ray crystallography. On the other hand, purified, misfolded and inactive molecule displays a decrease in α-helical content to 24%, accompanied by an increase in ß-sheet up to 42%, indicative of extensive changes in the conformation of the protein.

# 1. INTRODUCTION

Acetylcholinesterase (AChE) (EC 3.1.1.7) hydrolyzes acetylcholine at the post-synaptic membrane in the neuromuscular junction, while butyrylcholinesterase (BuChE) (EC 3.1.1.8), a serum-soluble cholinesterase of unclear function, hydrolyzes preferentially butyrylcholine. AChE and BuChE are distinguished by their selective inhibition with 1,5bis(4-allyldimethylammoniumphenyl)pentane-3-one dibromide(BW284C51) and tetraisopropylpyrophosphoramide (iso-OMPA), respectively. The molecular polymorphism, protein subunit organization and other properties of these cholinesterases have recently been reviewed (Chatonnet and Lockridge, 1989; Taylor, 1991). Several studies have shown that administration of fetal bovine AChE to animals prior to exposure to the organophosphorus (OP) poisons protected the animals from their toxic effects (Wolfe et al., 1987; Raveh et al., 1989; Ashani et al., 1991). Thus, the therapeutic potential of AChE against OP poisoning has triggered efforts to clone and express the human AChE in large quantities for further evaluation.

Recently, a cDNA prepared from adult basal ganglia cells encoding hAChE catalytic subunit was isolated and cloned (Soreq et al., 1990). The deduced amino acid sequence of the mature enzyme is 583 residues in length and contains three putative glycosylation sites, three disulfide bonds and an additional cysteine residue at the C-terminus ($Cys^{580}$), which is apparently involved in inter-subunit disulfide linkage formation, similar to that demonstrated for *Torpedo* AChE (Sussman et al., 1991). Synthetic mRNA, generated from the cDNA *in vitro*, was translated in microinjected oocytes into catalytically active enzyme. The enzyme produced in oocytes exhibited biochemical properties similar to the mature enzyme as manifested by substrate inhibition and sensitivity to the specific AChE inhibitor BW284C51 (Augustinsson, 1963). hAChE was cloned and expressed in the human embryonic kidney line 293, with a plasmid vector under control of the cytomegalovirus IE gene enhancer-promoter (Velan et al., 1991a; Velan et al., 1991b). The rhAChE secreted into the medium was shown to contain a mixture of active monomers, dimers and tetramers, or active monomers alone when $Cys^{580}$ was substituted by Ala.

In this final report, covering the period September 1990 to August 1993, we describe the construction of expression plasmids that support high-level production of rhAChE in *E. coli*, the *in vitro* reconstitution of the inactive protein from inclusion bodies into enzymatically active AChE, small- and large-scale purification methodology to obtain milligram quantities of the enzyme, as well as the biochemical and biophysical properties of the purified enzyme obtained from clone Sϕ930pMFL-52Ser. The polypeptide produced by this clone contains two amino acid changes. The amino acid Met precedes the first naturally occurring Glu in the mature enzyme and a substitution of $Cys^{580} \rightarrow Ser^{580}$, which eliminates the cysteine residue involved in interdimeric disulfide formation.

## 2. EXPERIMENTAL PROCEDURES

### 2.1 Reagents

2.1.1 Chemicals: Acetylthiocholine, HEPES, 5,5-Dithiobisnitrobenzoic acid (DTNB), tetraisopropylpyrophosphoramide (iso-OMPA), 1,5-bis(4-allyldimethylammoniumphenyl) pentane-3-one dibromide (BW284C51), Tris, urea, glutathione-oxidized (GSSG), polyethyleneglycol (PEG), glucose, L-arginine, tetramethylammonium chloride (TMAC), ethylenediaminetetraacetic acid (EDTA), dithioerythriol (DTE), acrylamide, sodium dodecylsulfate (SDS), Coomassie Blue R, butyrylthiocholine, ampicillin (Amp), tetracycline (Tet), sodium phosphate, sodium acetate, glycine and CNBr were purchased from Sigma Chemical Co., St. Louis, MI, U.S.A. Guanidinethiocyanate (GTC) and limited amounts of urea were from Fluka Chemical AG, Switzerland; DEAE-Sepharose and cyanogen bromide activated Sepharose 4B from Pharmacia, Sweden; 1-methyl-9[Nß-(ε-aminocaproyl)-ß-aminopropylamino]acridinium bromide (MAC) was obtained from the Weizmann Institute, Rehovot, Israel. CuZn superoxide dismutase (SOD) and recombinant human growth hormone (rhGH) are products of Bio-Technology General, Israel. For large scale refolding of rhAChE, GSSG was purchased from Boehringer Mannheim, Germany. Formic acid, ammonium carbonate and trifluoroacetic acid were from Merck. [$^{32}$P] labeled γ-ATP and [$^{32}$P]-αCTP were from Amersham, U.K.; ATP, GTP, CTP and TTP (nucleotide triphosphates) were from Boehringer Mannheim.

2.1.2 Enzymes: Restriction endonucleases, $T_4$-ligase, polynucleotide kinase and *E. coli* DNA polymerase (Klenow fragment) were purchased from New England Bio-Labs Inc., MA, U.S.A. Erythrocyte-derived hAChE, lysozyme and trypsin were from Sigma Co., U.S.A. Endoproteinase Glu-c (V-8) and Lys-c were purchased from Boehringer Mannheim, Germany.

### 2.2 Equipment

Enzyme activities, protein and turbidimetric determinations, and UV spectra were performed using PU8700 or PU8720 UV/visible spectrophotometer (Phillips). Fraction collector, column and monitoring units were from Pharmacia, Sweden. Densitometric analysis was made with a model 620 Vidio densitometer, Bio-Rad Laboratories, Inc. Circular dichroism was carried out with a Jasco 500 spectropolarimeter. Fluorescence spectroscopy was performed with a Jasco spectrofluorometer, model FP770. HPLC analysis was carried out with a Kontron instrument, equipped with HPLC pumps models 420 and 422S, detector model 430 and oven controller model 480, linked to a PC computer. The column used for separation was Aquapore AP-300 (250x4.6mm, 7μm particles) from Brownlee Labs. Bench-top fermentation was carried out in a New Brunswick 2L bioreactor and scaled-up in a 750L Bioengineering bioreactor (Switzerland). Pilot-scale concentrations and dialyses were carried out with a Filtron ultrafiltration system equipped with 10K membrane stack (3 layers of 5 feet filtration area).

## 2.3 Construction of expression vector

2.3.1 Construction of plasmid containing native rhAChE DNA sequence: A 4Kb DNA fragment harboring the hAChE was isolated from plasmid GEM-7 (Soreq et al., 1990) harboring the DNA sequences shown in Figure 1; this plasmid was kindly provided by Prof. H. Soreq (the Hebrew University, Jerusalem). The 2200bp AChE sequence that resides on the distal part of the 4Kb segment and is flanked by EcoRI-XhoI sites was isolated and ligated into pBR322 cleaved with EcoRI-SalI restriction endonucleases. The resulting plasmid containing the AChE was cleaved with NdeI and NaeI, and the larger of the two fragments generated by the cleavage was isolated from the agarose gel. A synthetic oligonucleotide containing complementary restriction sites (Figure 2) was ligated to the purified fragment. The synthetic oligonucleotide contained the initiation codon ATG and the sequence of AChE starting from base number 253 to base 298 (Soreq et al., 1990), which corresponds to the beginning of the mature protein after processing the sequences encoding leader peptide upstream of base number 253. The resulting plasmid pAIF-2 (Figure 2A) was cleaved with AatII (at base number 2128), made blunt end by DNA polymerase (Klenow fragment) and then cleaved with NdeI. The 1875bp fragment generated by these restriction enzymes was purified from agarose gel and ligated into appropriate expression vectors. In these constructs we have placed the hAChE sequence under control of the constitutive deo P promoters (Fischer et al., 1990) or the thermoinducible $\lambda P_L$ promoter (Hartman et al., 1986). The corresponding expression plasmids containing the authentic GC-rich stretches in the 5'-end of the hAChE-DNA sequence were designated pAIF-4 and pAIF-11, respectively (Figure 2A).

2.3.2 Manipulation of the 5'-end GC-rich sequence: The GC-rich sequence at the 5'-end of the hAChE was substituted with A or T in degenerated codons, such that the amino acid sequence was not altered. A synthetic oligonucleotide (Figure 2B) flanked by NdeI-NcoI containing 24 base substitution was ligated to pAIF-4 and pAIF-11. The resulting expression plasmids, driven by the thermoinducible or constitutive promoters, were designated pAIF-34 and pAIF-51, respectively. In plasmid pAIF-51, the $Amp^R$ gene was replaced with the $Tet^R$ gene to yield pAIF-52 (Figure 3). Replacement of $Amp^R$ by $Tet^R$ gene in the expression vector pAIF-52 stabilized the plasmid, and expression of rhAChE was maintained at the same level for more than 40 generations.

2.3.3 Substitution of $Cys^{580}$ by Ser: Three intrasubunit disulfide bonds can be deduced from the DNA sequence (Soreq et al., 1990) and from the three-dimensional structure of the *Torpedo* AChE (Sussman et al., 1991). The C-terminus cysteine residue of *Torpedo* AChE participates in the intersubunit disulfide bridge between two subunits (MacPhee-Quigley et al., 1986). Since the C-terminus cysteine residue of human and *Torpedo* AChE are positioned three amino acids upstream of the last amino acid, we predicted that $Cys^{580}$ (the C-terminus) of hAChE is involved in generating a disulfide bridge between two subunits and is not required for maintaining the protein integrity. Thus, this cysteine was replaced by serine by cleavage of plasmid pAIF-52 with SacI and XbaI and insertion of a synthetic oligonucleotide flanked by complementary sites that contained the serine codon TCA instead of the cysteine codon TGC in the authentic

sequence. The synthetic double-stranded oligonucleotide is shown below.

```
5' -       CCTACATGGTGCACTGGAAGAACCAGTTCGACCACTACAGCAAGCAGGATC

3' - TCGAGGATGTACCACGTGACCTTCTTGGTCAAGCTGGTGATGTCGTTCGTCCTAG
     sacI

        TGC
     -GCTCATCAGACCTGTGAT3'
     -CGAGTAGTCTGGACACTAGATC
                          xbaI
```

The resulting plasmid was designated pMFL-52Ser (Figure 3). Bacterial hosts and plasmid constructs are presented in Table 1.

## TABLE 1

## PLASMID CONSTRUCTS

| PLASMID | RESISTANCE MARKER | PROMOTER UPSTREAM TO AChE | RIBOSOMAL BINDING SITE | AChE SEQUENCE MODIFICATIONS |
|---|---|---|---|---|
| pAIF-2 | Amp | none | none | none |
| pAIF-4 | Amp | $\lambda P_L$ (thermoinducible) | λcII | none |
| pAIF-11 | Amp | deoP (constitutive) | deo | none |
| pAIF-34 | Amp | $\lambda P_L$ | λcII | 24 G and C to A and T (Figure 2) |
| pAIF-51 | Amp | deoP | deo | 24 G and C to A and T |
| pAIF-52 | Tet | deoP | deo | 24 G and C to A and T |
| pMFL-52Ser | Tet | deoP | deo | As pAIF-52 and $Cys^{580} \rightarrow Ser$ |
| pMIF-35Ser* | Amp | $\lambda P_L$ | λcII | As pAIF-34 and Cys→Ser |
| pMFEG-8 | Tet | deo | deo | 43 a.a. deletion C-terminus |
| pMFE-5234 | Tet | deo | deo | $Cys^{237}$ and $Cys^{272} \rightarrow Ser$ |
| pMFEG-89 | Tet | deo | deo | N-terminus and C-terminus deletions of 30 and 43 a.a., respec. |
| *E. coli* Hosts | | | | |
| Strains | Genotype | | | |
| A4255 | F-λcI$_{857}$, ΔH$_1$BamH, bio | | | |
| Sϕ930 | Δdeo, deoR-7. clmA, Δlac, udp, upp, ton, thi (Fischer et al., 1990) | | | |
| MC1061 | araD139, Δ (ara-leu)7696, galE15, galK16, Δ(lac)x74 rspl (Str), hsd22 ($r^-m^+$)deoC, mcrA, mcrB1 | | | |

*The marked construct was not analyzed

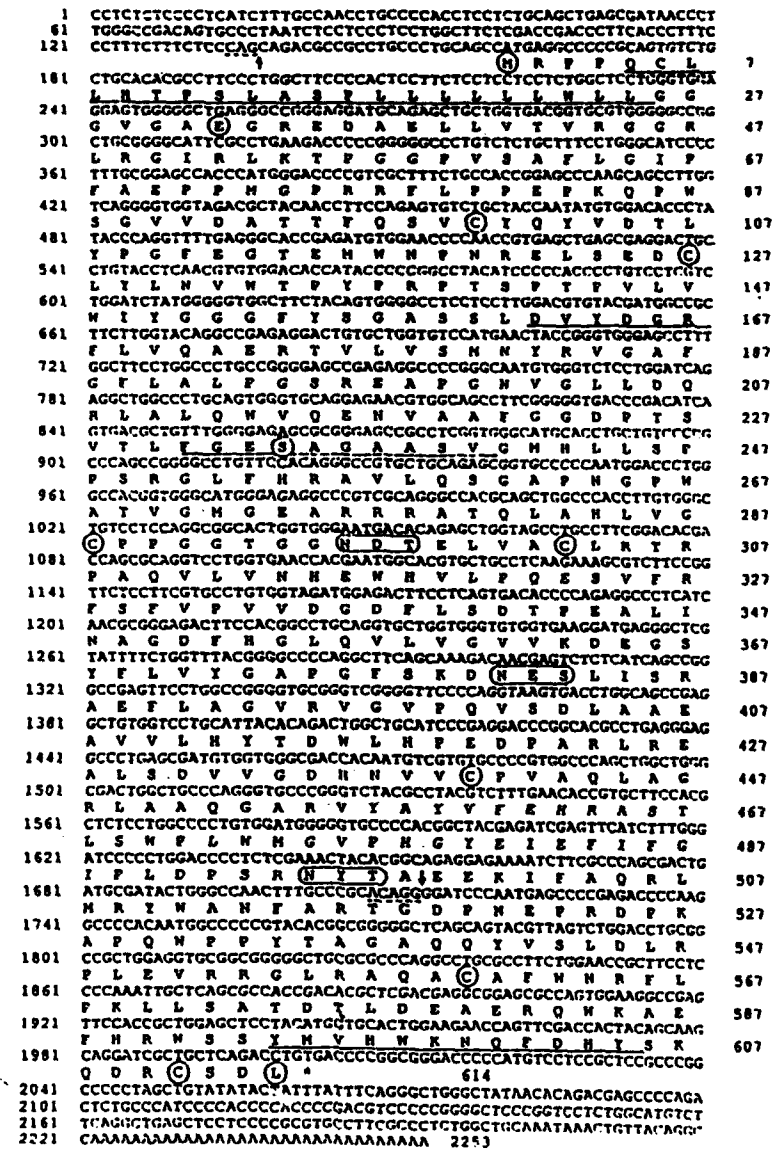


*Figure 1: Nucleotide and amino acid sequence of hAChE as published by Soreq et al., 1990. The single-letter code was used to denote individual amino acids. See the cited reference for explanation of boxed, underlined sequences and arrows.*

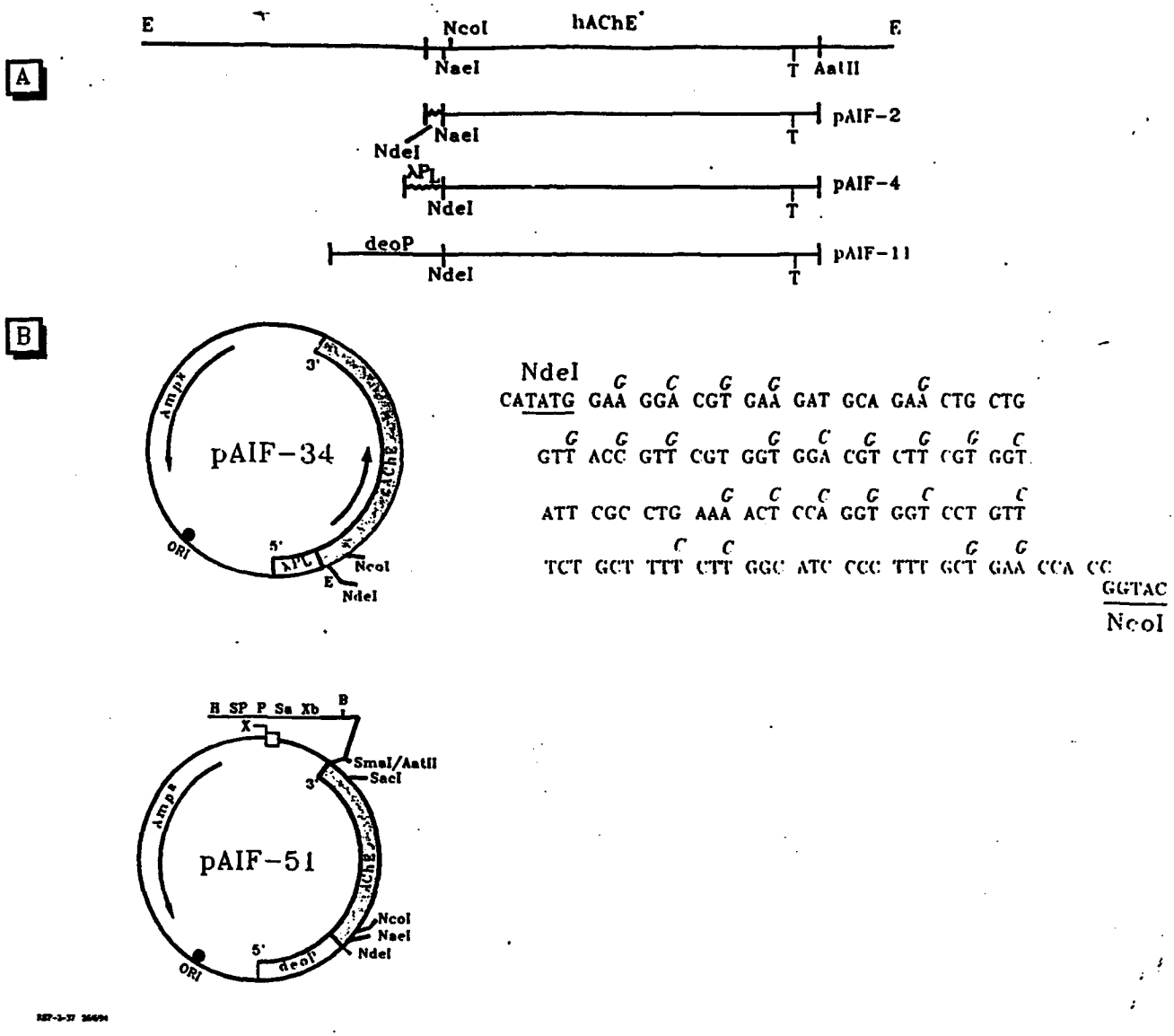
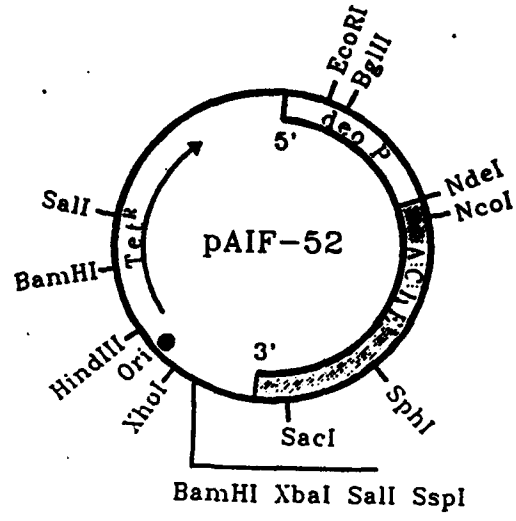



*Figure 2: Schematic presentation of rhAChE harboring plasmids*

*A: From top to bottom: A linear presentation of the coding sequences for AChE and respective promoters cloned in pBR322. 4kb; fragment harboring the hAChE cDNA. pAIF-2; intermediate construct which contains a synthetic linker harboring ATG translation initiation codon and the authentic, GC-rich, DNA sequence starting with the triplet GAG, encoding Glu, the first amino acid of mature hAChE protein, pAIF-4 and pAIF-11 are expression vectors driven by the thermoinducible $\lambda P_L$ and the constitutive deoP promoter, respectively. The NdeI site (CATATG) contains translation initiation codon (underline). T, indicates translation stop codon located 120bp upstream to AatII.*

*B: pAIF-34 and pAIF-51 plasmid with either $\lambda P_L$ or deoP promoters and a synthetic oligonucleotide replacing sequence between NdeI-NcoI site. The synthetic oligonucleotide sequence is shown on the right. The GC (with top letters) nucleotides substituted by A.T. Restriction cleavage site E, H, Sp, P, Sa, Xb, B, and X are EcoRI, HindIII, SspI, PstI, SalI, XbaI, BamHI and XhoI, respectively.*



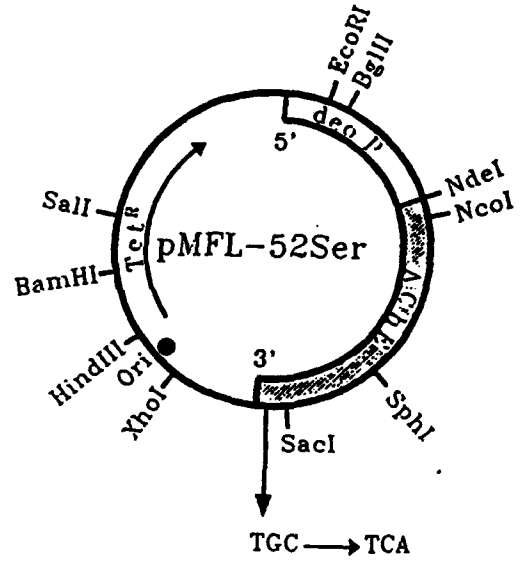




*Figure 3: Schematic presentation of expression plasmids. pAIF-52 contains the native amino acid sequence. pMFL-52Ser is identical to pAIF-52 with the exception that $Cys^{580}$ was replaced by Ser. The three-letter codon for cysteine, TGC, is replaced by the three-letter codon for serine, TCA.*

2.3.4 Construction of C-terminus truncated rhAChE: The C-terminus truncated rhAChE was constructed by cleavage of plasmid pMFL-52Ser with NotI and SacI endonucleases to remove a segment of double-stranded DNA located between nucleotides 1799 and 1935 (numbering according to sequence published by Soreq et al., 1990, PNAS 87:9688). A synthetic double-stranded oligonucleotide shown below containing complementary ends for NotI and SacI sequences and a TAA termination codon one base pair upstream to the SacI site replaced the excised fragment. The new plasmid was designated pMFEG-8. A schematic outline of the plasmid constructed is shown in Figure 4. Dots above the single-letter bases denote base substitutions from GC→AT. These substitutions do not alter the proper amino acid sequence.

```
NotI                G  G  G         C     C
                    .  .  .         .     .
5'GGCCGCTGGAGGTGCGTCGTGGTCTGCGCGCACAGGCAT
3'    CGACCTCCACGCAGCACCAGACGCGCGTGTCCGTA


GCGCCTTCTGGAACCGCTTCCTCCCGAAATTGCTCTAATGAGCT
CGCGGAAGACCTTGGCGAAGGAGGGCTTTAACGAGATTAC

                                          SacI
```

Plasmid pMFEG-8 was introduced to *E. coli* Sϕ930, selected on Luria broth (LB) Tet plates and cultivated in a 2L bioreactor in LB containing 12.5 μg/ml Tet to $OD_{660}$ of 25. The truncated rhAChE was expressed in the form of insoluble protein embodied in inclusion bodies.

Purification and solubilization of inclusion bodies and *in vitro* refolding into enzymatically active form was carried out as described for the full-length rhAChE derived from pMFL-52Ser.

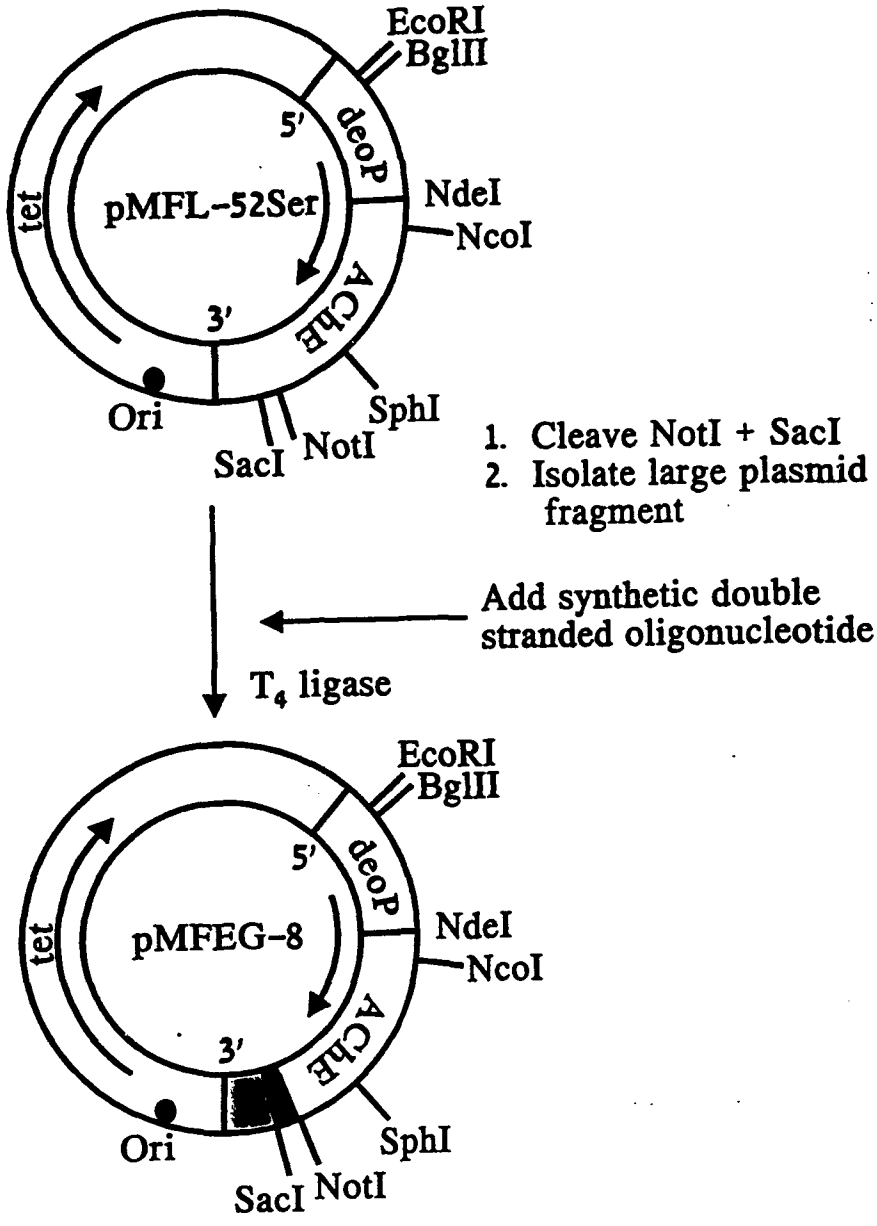



*Figure 4: Schematic presentation of plasmid pMFEG-8. This plasmid was derived from plasmid pMFL-52Ser by removing the sequence spanning between NotI and SacI recognition sites and insertion of a synthetic oligonucleotide with compatible ends. The dark area indicates the position of the synthetic oligonucleotide insertion. The dashed area shows the C-terminal region deleted from the parental plasmid.*

2.3.5 Construction of pMFEG-89: pMFEG-89 was constructed by the cleavage of pMFEG-8 with NdeI and NcoI to remove a DNA sequence spanning between nucleotides 252-342 (Soreq et al., 1990, PNAS 87:9688). A synthetic double-stranded DNA containing the initiation codon ATG (coding for Met) was ligated to the purified plasmid fragment. The synthetic oligo (shown below) encoded the amino acid sequence Met-Ala-Phe-Leu-Gly-Ile-Pro-Phe-Ala-Glu-Pro-Pro (from a.a. number 30 of the mature protein to a.a. 42), which lacks 30 a.a. from the N-terminus of the parental sequence.

```
          C  G                  G  G
NdeI      .  .                  .  .
TATGGCTTTTCTTGGCATCCCCTTTGCTGAACCACC
  ACCGAAAAGAACCGTAGGGGAAACGACTTGGTGGGTAC

                                  NcoI
```

The synthetic double-stranded oligonucleotide was used in construction of expression plasmid pMFEG-89 (Table 1, above). Dots above the sequence indicate base substitutions. The single bases above the dots show the naturally occurring bases in the sequence that were substituted without changing the proper amino acid sequence.

2.3.6 Construction of $Cys^{257}$ and $Cys^{272}$ to Ser substituted DNA of rhAChE: To replace cysteine residues 257 and 272 in the amino acid sequence of rhAChE, plasmid pMFL-52Ser was cleaved with DraIII and PmlI restriction endonucleases according to the recommendation of the manufacturer (Boehringer Mannheim). This cleavage removed a fragment of 99 base pairs that span from base number 1014 to 1113 (number according to the published sequence, Soreq et al., 1990). The endonuclease cleaved plasmid was subjected to agarose gel electrophoresis (1.2% low melt agar) and the large linear fragment extracted from the agarose (the 99bp cannot be detected on agarose gel). Oligonucleotides #4713 and #4825 were kinased with *E. coli* polynucleotide kinase prior to annealing. Four synthetic single-stranded oligonucleotides (#5116, 4713, 4825 and 5515) were annealed as shown below, and the resulting double-stranded oligos were ligated. The 99/102 bp double-stranded synthetic oligonucleotide flanked by DraIII complementary bases at the 5'-end and PmlI (blunt) at the 3'-end was ligated to DraIII-PmlI cleaved pMFL-52Ser isolated from agarose. The ligated product was introduced by electrophoration (Gene Pulsar apparatus, Bio-Rad, U.S.A.) to *E. coli* MC1061. $Tet^R$ transformants obtained on LB agar plates containing 12.5 μg/ml tetracycline were subjected to colony hybridization with $^{32}P$ labeled oligo #5515 to identify clones harboring the synthetic linker. Figure 5 is a schematic presentation of the procedure used to obtain the new plasmid, designated pMFE-5234. Note that the synthetic linker contains two base substitutions in addition to those used for Cys-Ser replacements. These substitutions did not alter any other amino acid in the protein sequence and were introduced to create a new restriction endonuclease site KpnI.

Synthetic oligonucleotides used to construct plasmid pMFE-5234:

```
        DraIII
          ↓      ┌─┐
                 |G|          C  T
                 └─┘
5'      GTGGGCTCTCCTCCAGGCGGTACCGGTGGGAATGACACAGAGCTGGTAGCC   #5116
3'    GAACACCCGAGAGGAGGTCCGCCATGGCCACCCTTACTGTGTCTCGA       #4713
                           KpnI

          ┌─┐                                          PmlI
          |G|                                           ↓
          └─┘
        TCTCTTCGGACACGACCAGCGCAGCTGCTGGTGAACCACGAATGGCAC     #4825
CCATCGGAGAGAAGCCTGTGCTGGTCGCGTCGACGACCACTTGGTGCTTACCGTG     #5515
```

*Boxed letters indicate Cys-Ser substitutions denoted by the underlined triplet TCT. Upper letters show the original base in the native cDNA sequence of rhAChE. The newly created KpnI site is marked.*

## 2.4 Cultivation

2.4.1 Media and growth: 2-50ml cultures were grown in LB broth supplemented with M9 salts, 0.1% glucose and 100μg/ml ampicillin or 12μg/ml tetracycline, depending on the culture used. Clones Sϕ930pMFL-525Ser and Sϕ930pAIF-52 were grown at 30°C for 16-18h, harvested by centrifugation for 10 min at 10,000 rpm in a Sorvall refrigerated centrifuge, washed with 50mM Tris-HCl, pH 8.0, and inclusion bodies were prepared as described below. Clone A4255 pAIF-34 (the thermoinducible expression system) was grown to $OD_{660}$ of 0.8 at 30°C. To induce expression of rhAChE, the temperature was elevated to 42°C and growth was extended for an additional 2h. The culture was harvested by centrifugation in a Sorvall refrigerated centrifuge for 10 min at 10,000 rpm. Cell lysates containing about 600μg of protein in 100μl were prepared by NaOH-SDS lysis solution (Hartman et al., 1986), boiled for 10 min and 15μl was applied per slot of SDS-PAGE. Following electrophoresis at room temperature, the gel was stained with Coomassie Brilliant Blue R.

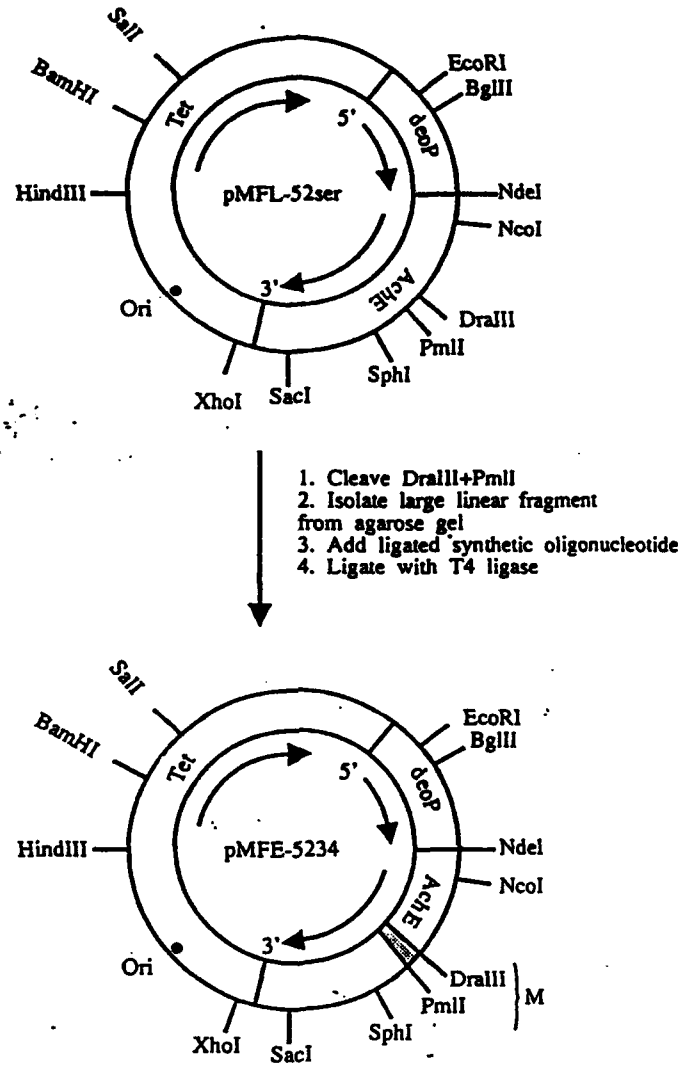



*Figure 5: Schematic presentation of construct containing $Cys^{257}$ and $Cys^{272}$ substitution to Ser. M: denotes the 99 bp synthetic linker flanked by DraIII and PmlI endonuclease recognition sites that harbor the Cys→Ser codon changes.*

2.4.2 Cultivation in bioreactors: Initial optimization for biomass production was carried out in a 2L bioreactor containing 20g/L of NZ-amine B (Sheffield Products, NY, U.S.A.), 10g/L yeast extract (Difco), 10mg/L tetracycline (Sigma) and 1.5g/L glucose. The pH of the media was adjusted to 7.2-7.3 and maintained automatically by a pH controller with 10% $NH_4OH$. The culture was harvested at an optical density of 23-25 at 660nm. Cultivation temperature was 30-37°C.

Further scale-up was accomplished by seeding the 750L bioreactor with Sϕ930pMFL-52Ser (seed culture) grown in a 35L bioreactor (Bioengineering) to $OD_{660}$ of 15-16. The seed culture was diluted into 500L media of the larger vessel to yield an $OD_{660}$ of 0.06-0.08. The culture was grown at 30°C or 37°C and harvested when the culture reached an $OD_{660}$ of 23-25.

## 2.5 Bench scale processing

2.5.1 Inclusion body purification and solubilization: To isolate inclusion bodies, 10g of packed cells were suspended in 100ml of 50mM Tris-HCl, pH 8.0, 10mM EDTA and 10μg/ml of lysozyme. The suspension was incubated for 2h at room temperature, sonicated intermittently for 5 min to disrupt cells and centrifuged for 15 min at 15,000 rpm in a Sorvall refrigerated centrifuge. The pellet was resuspended in 100ml of distilled water and stirred for 30 min. After centrifugation, the pellet was suspended in 10mM Tris-HCl, pH 8.0, containing 4M urea and stirred at room temperature for 1h. The inclusion bodies were collected by centrifugation for 30 min at 15,000 rpm, resuspended in 100ml distilled water and stirred at 4°C for 16-18h. Inclusion bodies were then collected by centrifugation and resuspended in 10ml of 10mM Tris-HCl, pH 8.0. Solid guanidinethiocyanate (GTC) was added to a final concentration of 5.5M in 25ml. After solubilization of inclusion bodies, the solution was brought to pH 8.6 with 1M NaOH and was stirred for 16-18h at room temperature. The GTC-solubilized inclusion bodies were centrifuged at 15,000 rpm for 30 min at room temperature to remove undissolved matter and then were diluted 1:10 into 10mM Tris-HCl, pH 8.6, containing 8M urea.

2.5.2 Reconstitution of rhAChE *in vitro*: The 1:10 diluted rhAChE in 8M urea was diluted into a refolding solution to yield a final concentration of 30-100μg/ml protein. The refolding solution contained 0.5M L-arginine, pH 10.0, 1M tetramethylammonium chloride, 0.3mM GSSG and 0.3% polyethyleneglycol (PEG) 4000 at 4°C. After the addition of denatured rhAChE, the solution was incubated for 24-72h at 4-8°C, dialyzed against 5mM L-arginine, pH 10.0, containing 1mM EDTA, for 16-18h at room temperature. Acetylcholinesterase activity was determined according to Ellman et al. (1961) at 412nm using acetylthiocholine as substrate. The assay mixture contained 0.1M HEPES, pH 8.0, instead of phosphate buffer. We have introduced this modification because no spontaneous hydrolysis of acetylthiocholine is observed in HEPES.

2.5.3 Partial purification of active rhAChE: The active rhAChE was subjected to Q-Sepharose column chromatography. The column was equilibrated with 20mM HEPES, pH 8.0, and the active rhAChE was eluted with NaCl gradient (0-0.5M). Fractions containing active AChE were pooled and precipitated with ammonium sulfate at 45% saturation, dialyzed against 20mM HEPES, pH 8.0, and applied to MAC-Sepharose 4B prepared according to Dudai et al. (1972). Active rhAChE was eluted from the affinity matrix with 20mM HEPES, pH 8.0, containing 2M NaCl.

## 2.6 Process development scale-up

2.6.1 Purification of inclusion bodies: To elucidate the process for large scale preparations, we present the step-by-step procedure that corresponds to the results summarized in Table 9. The bacterial biomass (6kg of slurry) was resuspended in 50mM Tris-HCl, pH 8.0, containing 10mM EDTA and 10mg/L lysozyme in a final volume of 50L. The suspension was incubated for 16h at 4°C and disrupted in a bead mill (Dyno-mill). Inclusion bodies were collected by continuous centrifugation in a Sharples centrifuge at maximum speed. The pellet was resuspended in 50L of distilled water, stirred for 30 min and centrifuged as described above. The water-washed inclusion bodies were resuspended in 25L of 10mM Tris-HCl, pH 8.0, containing 4M urea and stirred for 2h at room temperature. Inclusion bodies were collected by centrifugation and resuspended in 10mM acetate buffer, pH 5.2-5.4, and incubated while stirring at 4°C for 16-18h. The final pellet of inclusion bodies obtained after centrifugation contained about 70% rhAChE as determined by SDS-PAGE (Laemmli, 1970) on 15% acrylamide gels stained with Coomassie Brilliant Blue R.

2.6.2 Solubilization of inclusion bodies: To solubilize the inclusion bodies, 10ml of 6M GTC containing 10mM Tris-HCl, pH 8.6, was added per gram of pellet and stirred for 2-4h at room temperature. The dissolved protein solution was adjusted to pH 9.5-10.0, with 10M NaOH and stirred for 2h. DTE was added thereafter to a final concentration of 20mM; the pH was then brought to 5.4 with concentrated acetic acid and the solution was stirred for 16h at room temperature. The final volume of 2.2L contained 70.4g protein (32mg/ml).

2.6.3 Refolding: The refolding of rhAChE into enzymatically active form consisted of the following steps: The protein dissolved in GTC was diluted 23-fold into 10mM Tris-HCl, pH 8.6, containing 8.5M urea to a final volume of 50L and incubated at room temperature for 24-48h. The solution was then diluted 10-fold into cold refolding buffer containing 50mM L-arginine, pH 10.0, 50mM tetramethylammonium chloride, 0.3mM GSSG and 0.3% PEG 3350, to yield a final volume of 600L. Refolding was performed at 8-10°C at a protein concentration of 100µg/ml in a temperature-controlled tank for 48h. The volume of 600L was reduced to 130L by concentration on a 10K membrane filter (Filtron Ultrafiltration system) and then dialyzed against 1000L of 10mM L-arginine, pH 10.0, using the same filtration/dialysis device. The concentrated material was incubated at room temperature for 5 days and regain of enzyme activity was monitored daily.

The volume of the solution containing active rhAChE was reduced to 25L by concentration on a 10K membrane filter as described above. The solution was adjusted to pH 8.1, with acetic acid, prior to loading onto a DEAE-Sepharose column. The column (113x400mm), containing 3.8L of resin, was equilibrated with 20mM Tris-HCl, pH 8.1, and the protein solution was loaded onto the column at a rate of 146ml/min. After washing with equilibration buffer, the column was further washed with 20mM Tris-HCl, pH 8.1, containing 0.175M NaCl. The active rhAChE was eluted with the same buffer containing 0.4M NaCl. The eluted protein (12.8L) was concentrated/dialyzed on a 10K membrane filter (Filtron) against 20mM HEPES, pH 8.0, containing 1mM EDTA, to a final volume of 2L.

2.6.4 Affinity chromatography: The ligand 1-methyl-9[Nß-(ε-aminocaproyl)-ß-aminopropylamino] acridinium bromide (MAC) was coupled to CNBr-activated Sepharose-4B (Dudai et al., 1972). The MAC-Sepharose affinity column (26x450mm) containing 80ml of packed resin was equilibrated with 20mM HEPES, pH 8.0. The protein was loaded onto the column, washed with 300ml of equilibration buffer and the bound inactive enzyme was eluted with the same buffer containing 0.2M NaCl. Pooled inactive rhAChE was further purified to apparent homogeneity by Q-Sepharose chromatography, equilibrated with 10mM (3-[cyclohexyl amino]-1-propanesulfonic acid (CAPS) buffer pH 10.0, and eluted with 250mM NaCl in the same buffer. The highly active rhAChE was eluted from the MAC column with 0.2M L-arginine, pH 10.0, containing 2.5mM EDTA. Due to the limited capacity of the affinity column, three consecutive runs were needed to affinity-purify the entire batch. The active enzyme fractions eluted with L-arginine were pooled, precipitated with 45% saturation of ammonium sulfate, resuspended in 20mM HEPES, pH 8.0, containing 50mM NaCl, 2.5 mM EDTA, and dialyzed against 100 volume of the same buffer with three changes.

## 2.7 Analytical methods

2.7.1 Determination of AChE activity: Enzyme activity was determined at 412nm at 25°C and monitored on a Phillips spectrophotometer, model PU8700. One unit of activity corresponds to the hydrolysis of 1µmol acetylthiocholine/min. The enzyme assay was according to Ellman et al. (1961) in a final volume of 1ml.

2.7.2 Stability determination: The purified rhAChE was diluted into 0.1M HEPES, pH 8.0, in the presence or absence of 0.5mg/ml bovine serum albumin (BSA). The diluted enzyme was incubated at room temperature (22-24°C) or at 37°C and aliquots were removed to determine the remaining activity. To determine the pH-stability profile of the enzyme, both purified rhAChE and human erythrocyte AChE were diluted into 0.1M of each of the following buffers: sodium citrate pH 4.0, Tris-acetate pH 5.0, sodium phosphate buffer pH 6.0 and 7.0, Tris-HCl pH 8.0, Tris-glycine pH 9.0, bicarbonate pH 10.0 and 11.0 , all containing 0.5mg/ml BSA. The diluted enzyme was incubated at 4-5°C for a period of 10 days. Aliquots were removed at time intervals to determine the remaining AChE activity.

2.7.3 Isoelectric focusing (IEF): The pI of rhAChE was determined by IEF analysis in

polyacrylamide $T_5C_3$ gel, containing pharmalyte solutions in a pH range of 3.0–10.0 (Pharmacia), with and without 6M urea and 10% glycerol. Purified rhAChE samples of 6μg per slot were applied to the 10% acrylamid gel. The protein pI markers used in this analysis were (1) isoforms of recombinant human Cu/Zn–SOD (Bio–Technology General) with pI 4.85, 4.95 and 5.16; (2) rhGH pI 5.1, and (3) myoglobin from horse heart with pI values of 6.8 and 7.2. Electrophoresis was at 4400 v/h at a controlled temperature of 10°C. Following fixation, the gel was stained with Coomassie Brilliant Blue. The pI value of rhAChE was derived from the pI calibration curve shown in Figure 23B.

2.7.4 Determination of protein concentration: Protein determination was according to Macart and Gerbaut (1982). This method is based on the effect of SDS, which equalizes the reactivity of various proteins toward the Coomassie Brilliant Blue agent, leading to an equal sensitivity of the dye for all proteins.

2.7.5 Amino acid analysis: Amino acid composition of the rhAChE was determined after hydrolysis in the gas phase. The amino acids were separated by chromatography on an HPLC–attached cation exchange column and subjected to post–column derivatization by dabsyl chloride according to the procedure of Moore and Stein (1951) with the modification introduced by Knecht and Chang (1986). Two calculation methods were used for the determination of protein concentration: (a) nmol amino acid/average nmol of protein – which was determined by dividing the observed amount (in nmol) of each amino acid (Table 11) by the theoretical residue number; (b) nmol amino acid/Σnmol of the 18 amino acids, excluding cysteine and tryptophan.

2.7.6 UV spectroscopy: The spectrum was obtained on a Phillips scanning spectrophotometer model PU8720, and Hewlett Packard diode array spectrophotometer model 8452A, on sample No. 1 (Table 10). The data in each spectrum have been corrected for the residual background and light scattering by subtracting the absorbance obtained at 350nm from the absorbance values at 280 and 250nm.

2.7.7 CD spectroscopy: CD spectra were obtained on a Jasco 500 spectropolarimeter. The spectra shown are the average of four scans. Scanning speed was a compromise between maximal resolution and minimal denaturation of the sample during the measurement. The speed provided four readings/nm.

Two spectral domains were investigated (Strickland, 1974): The near UV (260–340nm) provided data about the tertiary structure of the protein, and the far UV (200–245nm) provided data about its secondary structure. Cylindrical quartz cuvettes, having pathlengths of 1 and 0.1cm for near and far UV measurements, respectively, were used. Estimations of the secondary structure from the far UV spectra were done by a computer program (Brumfeld and Miller, 1988), according to the method described by Chang et al. (1978). The estimated errors resulting both from experimental deviations and the least squares analysis were 2% for the α–helix and 4% for other structures The calculations were based on the cDNA–deduced amino acid sequence (Soreq et al., 1990), i.e., 583 amino acids and a molecular weight of 64,700 daltons.

2.7.8 Fluorescence spectroscopy: The fluorescence emission spectrum was obtained at 25°C on a Jasco spectrofluorometer model FP770, under the following conditions: λex = 295nm, excitation and emission slits – 5nm, sensitivity – 10. The fluorescence efficiency, $R_{Trp}$ (Cowgill, 1968) for rhAChE, was derived from the equation $R_{Trp} = h_A/h_T \times A_T/A_A$, where $h_A$ and $h_T$ are the peak heights of the emission spectra of the rhAChE and the Trp model compound (N-acetyltryptophanamide), respectively, and $A_A$ and $A_T$ are their absorbencies (determined in this case at 280nm).

2.7.9 Peptide mapping: Purified rhAChE harboring the $Cys^{580} \rightarrow Ser^{580}$ substitution was subjected to proteolytic digest with three different proteases. The cleavage product was then analyzed by HPLC as described below.

2.7.10 Tryptic digest: 3mg of rhAChE in 100µl of 50mM glycine-NaOH, pH 10.6, 1mM $CaCl_2$ and 8M urea was mixed with 300µl solution containing 50mM glycine-NaOH, pH 10.6, and 1mM $CaCl_2$. 20µl of trypsin (0.5mg/ml stock) was added, mixed and incubated at 25°C for 5h. A 25µl aliquot of the same trypsin solution was then added and the reaction was allowed to continue for 19h. 380µl of a solution containing 50mM glycine-NaOH, 1mM $CaCl_2$ and 8M urea pH 10.6, was added to yield a final urea concentration of 4M. To cleave the disulfide bond, 30µl of 1M DTT was added and the mixture incubated in boiling water for 1 min. After cooling to room temperature, 50µl of 2% trifluoroacetic acid (TFA) was added to acidify the mixture and a sample of 100µl was used for analysis.

The analysis was carried out with a Brownlee Labs Aquapore RP-300 column (250 x 4.6mm) using Kronton HPLC detector system. The gradient used for chromatography consisted of two solutions. Solution (a) contained 0.1% TFA in water and solution (b) contained 0.05% TFA in 50% acetonitrile and 50% water.

2.7.11 Endoproteinase Glu-c (V8) digest: 1mg of rhAChE in 50µl solution containing 25mM ammonium carbonate, pH 7.8 and 8M urea was mixed with 210µl of the same buffer without urea. The mixture was supplemented with 40µl of V8 protease (1mg/ml stock) and incubated for 16h at 25°C. The urea concentration was raised to 8M by adding 1.08mg of solid urea. 10µl of 1,4-dithiothreitol (DTT) (1M stock) was added, mixed and placed in boiling water for 1 min. After cooling to room temperature, 15µl of 2% TFA was added. A sample of 100µl was analyzed by HPLC. The column dimension and the gradient were the same as described for the tryptic digest analysis.

2.7.12 Endoproteinase Lys-C digest: The contents of the stock solutions (a) to (f), used in this analysis, are defined below. 90µl of solution (a) was first added to solution (c) containing the rhAChE mixed. 20µl of solution (f) containing the proteinase was then added and the mixture was incubated for 18h at 37°C with gentle shaking. After treatment with the proteinase, 54mg of solid urea was added and mixed until fully dissolved. A volume of 5µl of solution (d) was added and heated for 1 min at 100°C. The mixture was cooled to room temperature and 10µl of solution (c) was added. After centrifugation for 2 min in a bench top centrifuge, 100µl was used for HPLC analysis.

Stock solutions:

(a) 25mM Tris-HCl pH 8.5, containing 1mM EDTA (Sigma, USA)
(b) 8M urea containing 25mM Tris-HCl pH 8.5, and 1mM EDTA (Sigma)
(c) 2% Trifluoroacetic acid (TFA) (Merck)
(d) 1M 1,4-Dithiothreitol (DTT) (Sigma)
(e) 450µg of rhAChE in 20µl solution (b)
(f) 5µg of endoproteinase Lys-C in 20µl distilled water (Lys-C sequencing grade cat. # 1047825, from Boehringer Mannheim)

Solutions for HPLC: Acetonitrile - extra dry - 2.5L (Bio-Labs Laboratories); TFA - from Merck; $H_2O$ - double distilled water.
Solution (A): 0.1% TFA in water; solution (B): 0.085% TFA in 50% acetonitrile/water.

The $OD_{210}$ nm should be determined for solutions (A) and (B). If $OD_{210}$ of the two solutions differs by more than 0.010, then dilute solution (A) with water.

Column and detector system: Column - Brownlee Labs Aquapore RP-300 CO3-10A, 250 x 4.6mm with 7µm particles

Hardware: Kronton 430 detector
Kronton 450-MT data system
Kronton 480 column oven and controller
Kronton 360 autosampler
Kronton 420 and 422-S pumps
Kronton M-491 mixer

2.7.13 Chemical cleavage: Cleavage of rhAChE with CNBr, or with 88% formic acid, was carried out. However, upon acidification of the cleavage product, the peptides precipitated and could not be analyzed.

2.7.14 Standard procedures: DNA sequencing was performed according to the dideoxynucleotide incorporation by the method of Sanger et al. (1977). Restriction endonuclease cleavage and ligation were done according to the manufacturer's recommendations. SDS-PAGE was according to Laemmli (1970). AChE activity gels were performed as described by Karnovsky and Roots (1964). Amino acid sequence was determined with a protein microsequencer (Applied Biosystem 475A), based on the Edman degradation procedure (Edman, 1950; Tarr, 1977).

2.7.15 Immunoblots and colony hybridizations: Methods were as described by Maniatis et al. (1982). Transformation of bacterial cells with plasmid DNA was carried out by electrophoration according to the recommendaticns of the manufacturer (Bio-Rad, U.S.A.).

## 2.8 Synthesis

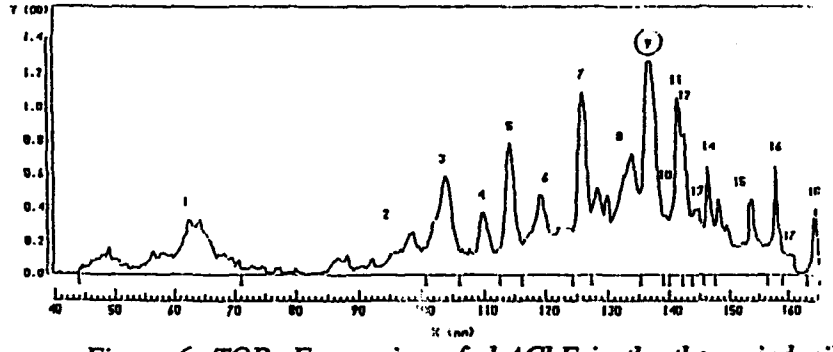
Oligonucleotide synthesis: Oligonucleotides are routinely synthesized at Bio–Technology General by automated solid phase phosphoramidite chemistry (McBride and Caruthers, 1983), using cyanoethyl phosphoramidite monomers. A Pharmacia gene assembler, capable of performing six synthetic cycles per hour, is used for the syntheses. At the end of each synthesis, the oligomer is cleaved from the solid support and deprotected by treatment with concentrated ammonia. Crude oligomers are usually purified by preparative acrylamide gel electrophoresis.

# 3. RESULTS

## 3.1 Effect of AChE DNA sequence manipulations on expression and refolding

3.1.1 Expression of rhAChE: Preliminary attempts to express hAChE gene by two different expression vectors, pAIF-4 and pAIF-11, under control of the $\lambda P_L$ or the deoP promoters respectively, were unsuccessful. The GC-rich sequences at the 5'-end of the gene might have generated secondary structures in the mRNA that are poorly accessible to ribosomes. Therefore, the high GC content at the 5'-end of the sequence was replaced by A or T at "wobble" positions which do not change the authentic amino acid sequence, as shown in Figure 2. Expression vectors harboring the deoP and the $\lambda P_L$ promoters and the modified sequence were introduced into *E. coli* Sϕ930 and A4255, respectively. Total cell lysates prepared in SDS-NaOH were analyzed on SDS-PAGE. Figure 6 shows that an intense protein band corresponding to a molecular weight of 62kD accumulates in the induced culture of A4255 pAIF-34 (lane 2). This protein band is not seen in uninduced control culture (Figure 6, lane 1). Western blot analysis, presented in Figure 7, revealed that the 62kD protein band immunoreacted with antibodies prepared against erythrocyte-derived authentic AChE and thus confirmed that the newly produced 62kD protein band represents a genuine AChE polypeptide. Densitometric analysis of lane 2 (Figure 6) aimed at determining expression level shows that the 62kD protein band, peak number 9, represents about 10% of the total cell protein. A similar expression level of rhAChE protein was obtained in clone Sϕ930pMFL-52Ser. In view of the simple growth conditions for expression of rhAChE clones harboring the deo promoter's driven system, we elected to use it routinely.

To determine the localization of expressed AChE, the A4255 pAIF-34 and Sϕ930pMFL-52Ser cultures were sonicated until 99% of the cells were disrupted and the soluble fraction was separated from insoluble matter by centrifugation. The nonsoluble fraction was resuspended in 1% SDS-NaOH, and 5μg of protein of each fraction was analyzed on SDS-PAGE. The electrophoretic analysis revealed that rhAChE was localized in inclusion bodies in both cultures and did not possess enzymatic activity. The 62kD protein band, observed on SDS-PAGE, was isolated and the amino acid sequence of the N-terminus was determined. The results of the sequencing confirmed the expected sequence of Met-Glu-Gly-Arg-Glu-Asp-Ala-Glu-Leu-Leu-Val.


| PEAK No. | POSITION | % FROM ORIGIN | RELATIVE MOBILITY | HEIGHT O.D. | WIDTH | AREA OD.% | REL. AREA % TOTAL |
|---|---|---|---|---|---|---|---|
| 1 | 47.1 | 22.4 | 0.17 | 0.310 | 14.25 | 3.407 | 11.57 |
| 2 | 71.0 | 33.2 | 0.42 | 0.270 | 7.70 | 2.149 | 7.30 |
| 3 | 93.9 | 44.2 | 0.61 | 0.570 | 5.14 | 1.809 | 6.24 |
| 4 | 109.9 | 39.2 | 0.54 | 0.389 | 5.32 | 1.268 | 4.21 |
| 5 | 119.5 | 34.0 | 0.57 | 0.799 | 1.99 | 1.595 | 5.26 |
| 6 | 129.1 | 99.5 | 0.62 | 0.199 | 5.06 | 2.137 | 8.09 |
| 7 | 136.1 | 86.4 | 0.86 | 1.099 | 1.87 | 2.062 | 6.84 |
| 8 | 152.7 | 72.5 | 0.71 | 0.739 | 5.31 | 3.910 | 12.98 |
| (9) | 157.1 | 97.6 | 0.75 | 1.270 | 2.47 | 3.207 | 10.65 |
| 10 | 157.6 | 100.0 | 0.77 | 0.369 | 0.76 | 0.362 | 1.20 |
| 11 | 161.5 | 198.9 | 0.79 | 1.069 | 1.33 | 1.452 | 4.82 |
| 12 | 162.8 | 101.2 | 0.77 | 0.959 | 1.09 | 0.748 | 3.12 |
| 13 | 166.8 | 105.1 | 0.81 | 0.649 | 1.75 | 0.712 | 2.36 |
| 14 | 176.4 | 107.9 | 0.82 | 0.640 | 1.15 | 0.782 | 2.69 |
| 15 | 181.9 | 112.1 | 0.86 | 0.469 | 5.09 | 2.295 | 7.65 |
| 16 | 190.9 | 118.1 | 0.91 | 0.449 | 1.15 | 0.771 | 2.56 |
| 17 | 160.1 | 120.7 | 0.93 | 0.149 | 2.11 | 0.303 | 1.01 |
| 18 | 164.5 | 125.9 | 0.96 | 0.109 | 0.96 | 0.372 | 1.30 |
| 19 | 166.6 | 126.9 | 0.97 | 0.060 | 1.91 | 0.110 | 0.37 |

*Figure 6: TOP: Expression of rhAChE in the thermoinducible clone A4255pAIF–34. Samples of total lysate and cells disrupted by sonication were analyzed in 15% SDS–PAGE.*
*Lane 1: Total cell lysate from uninduced culture; lane 2: total cell lysate from induced culture; lane 3: soluble fraction of sonicated culture; lane 4: insoluble fraction of sonicated culture;lane 5: molecular weight markers – from top to bottom (kD) – 97,66,47,30,20,14.4.*
*BOTTOM: Densitometric analysis of lane 2 shown on top. Peak number 9 corresponds to the 62kD rhAChE protein band. The numeric display on the right of the scan shows the data and the relative area, as percent, of the entire lane length.*

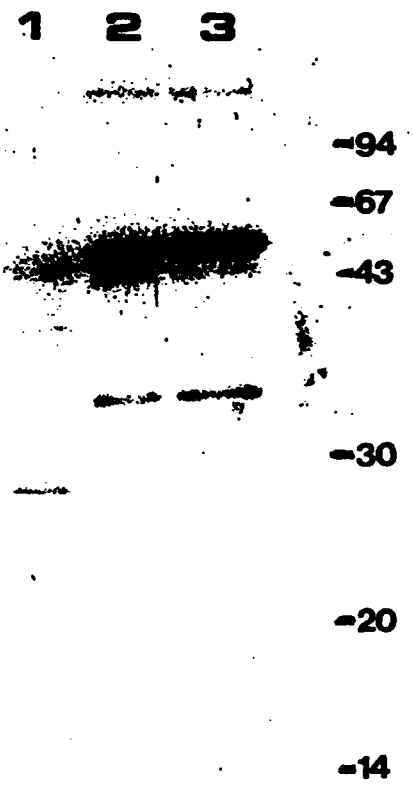



*Figure 7: Western blot of rhAChE. Total cell lysate and inclusion bodies isolated from clone Sϕ930 pMFL-52Ser were subjected to SDS-PAGE on a 15% acrylamide gel, blotted onto nitrocellulose paper and immunoreacted with human erythrocyte anti-AChE.*
*Lane 1: total cell lysate of control (host) Sϕ930; lane 2: total cell lysate of Sϕ930pMFL-52Ser; lane 3: inclusion bodies fraction isolate from pMFL-52Ser. The most intense immunoreactive band corresponds to the 62kD polypeptide of rhAChE. Numbers on the right are protein MW markers in kD.*

3.1.2 Enzyme activity of native and mutant ($Cys^{580} \rightarrow Ser^{580}$) rhAChE: Solubilized inclusion bodies purified from Sϕ930pAIF-52 (unaltered a.a sequence, i.e., wild-type or native) and Sϕ930pMFL-52Ser (mutant) culture were refolded and oxidized to obtain enzymatically active protein as described in the section on experimental procedures. Table 2 compares the rhAChE activities obtained with a construct containing the native amino acid sequence and with a mutant in which $Cys^{580}$ was replaced by Ser. The enzyme activity obtained with the $Cys^{580} \rightarrow Ser^{580}$ mutant was considerably higher than that obtained with the wild-type. A 71-fold increase in specific activity of the mutant AChE was obtained relative to the wild-type in the absence of GSSG, whereas a 32-fold higher specific activity of the mutant protein was noted in the presence of GSSG. The increase in recovery of enzyme activity after *in vitro* refolding of the mutant rhAChE was apparently related to the lower number of Cys residues in the molecule.

TABLE 2

ACTIVITY OF rhAChE AFTER REFOLDING AND OXIDATION

| rhAChE | GSSG | ACTIVITY (U/ml) | PROTEIN (mg/ml) | SPECIFIC ACTIVITY (U/mg protein) |
|---|---|---|---|---|
| Native | - | 0.007 | 0.025 | 0.28 |
| Native | + | 0.040 | 0.030 | 1.40 |
| Mutant | - | 0.580 | 0.029 | 20.10 |
| Mutant | + | 1.200 | 0.026 | 46.10 |

*Effect of mutation on activity of rhAChE after refolding and oxidation. Inclusion bodies purified from Sϕ930pAIF-52 that produce rhAChE with $Cys^{580}$ (native) and Sϕ930pMFL-52Ser that produce rhAChE with $Ser^{580}$ (mutant) were solubilized in 5.5M guanidinethiocyanate and subjected to renaturation in L-arginine refolding solution for 48 h at 4-6°C with and without GSSG. Enzyme activity was determined after removal of salts by dialysis. One unit equals the amount of enzyme which hydrolyzes 1 μmol of acetylthiocholine/min at 25°C. Accuracy of protein measurement was ±5%, and of activity ±2%.*

The zymogram of rhAChE on nondenaturing 6% polyacrylamide gel stained for enzymatic activity according to Karnovsky and Roots (1964) is shown in Figure 8. The zymogram revealed that the activity band generated by the native enzyme migrated slightly faster relative to the mutant harboring the $Cys^{580} \rightarrow Ser^{580}$ substitution.

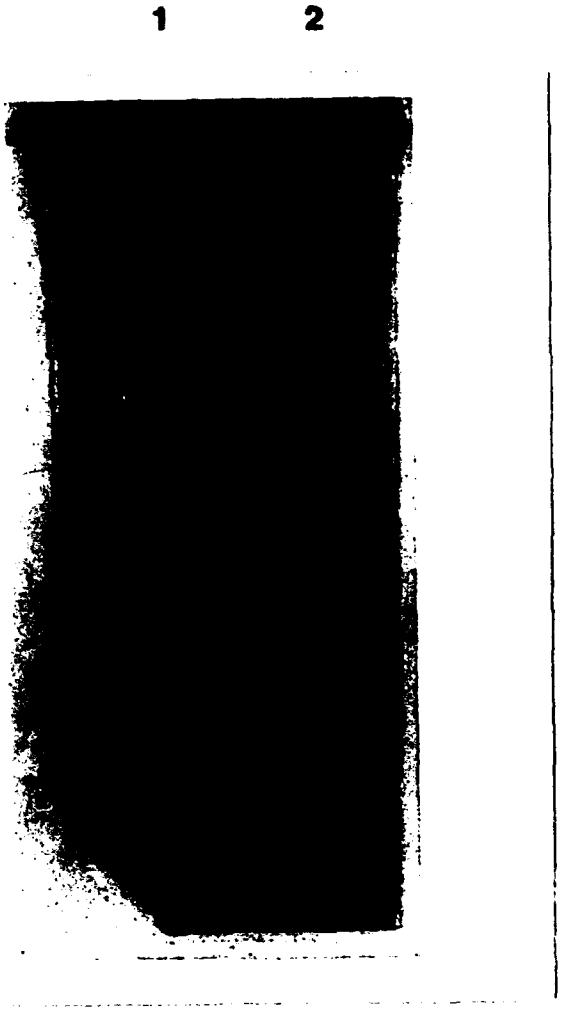



*Figure 8: Zymogram of rhAChE activity on 6% polyacrylamide gel. Lane 1: native rhAChE with $Cys^{580}$ reconstituted from inclusion bodies derived from clone Sϕ930pAIF-52. Lane 2: mutant rhAChE with $Cys^{580} \rightarrow Ser^{580}$ substitution reconstituted from inclusion bodies derived from clone Sϕ930pMFL-52Ser.*

3.1.3 C-terminus truncated rhAChE: The rhAChE that lacks the last 43 amino acids encoded by plasmid pMFEG-8 was expressed in host Sϕ930. The level of expression appears to be similar to that obtained with pMFL-52Ser (Figure 9). The truncated forms of the protein were expressed in the form of inactive aggregated polypeptides as inclusion bodies. Solubilization of inclusion bodies in guanidinethiocyanate followed by dilution into refolding buffer resulted in formation of enzymatically active truncated rhAChE. To evaluate the refolding efficiency of the new construct, we have subjected solubilized protein for both truncated and nontruncated rhAChE (derived from Sϕ930pMFL-52Ser) to refolding *in vitro* and compared the specific activities obtained. Table 3 summarizes the results of several such experiments. It is apparent that the truncated form of rhAChE, derived from Sϕ930pMFEG-8, yielded specific activities 2- to 3-fold higher relative to the nontruncated control. Thus, removal of 43 amino acids from the C-terminus improved refolding. Reconstitution of AChE derived from Sϕ930pMFE-5234 yielded extremely low activity, which we have considered as nonfunctional, since its specific activity was nearly 1500-fold lower, relative to the control. This result indicates that the two cysteine residues at position 257 and 272 in the a.a sequence are essential for structural maintenance of the catalytic subunit.

To determine if the removal of 43 amino acids affected the $K_m$, we determined the $K_m$ for the nonpurified enzyme using butyrylthiocholine and acetylthiocholine as substrates. Two such experiments revealed that the $K_m$ of the truncated rhAChE was 0.14mM and 0.106mM for butyrylthiocholine and acetylthiocholine, respectively. Figure 10 shows the Lineweaver-Burk plot for the $K_m$ determinations. The above preliminary results suggest that the elimination of 43 amino acids in the C-terminus of the enzyme has not impaired its catalytic activity.

To explore the possibility that the AChE molecule may be trimmed further at the N-terminus and thus further improve folding, plasmid pMFEG-89, which lacks the sequence encoding the first 30 amino acids at the N-terminus and 43 amino acid at the C-terminus, was expressed in *E. coli* host Sϕ930. Inclusion bodies isolated from a culture grown in a 2L bioreactor were subjected to *in vitro* refolding as described previously. No enzyme activity was detected after the refolding (Table 3). The lack of enzyme activity suggests that the first 30 amino acids at the N-terminus are important for the function of the enzyme. The two-dimensional scheme of the hAChE amino acid sequence based on the model for BuChE (Neville et al. 1992), presented in Figure 11, shows by the arrows indicated, the AChE molecules expressed by Sϕ930pMFEG-8 and pMFEG-89.

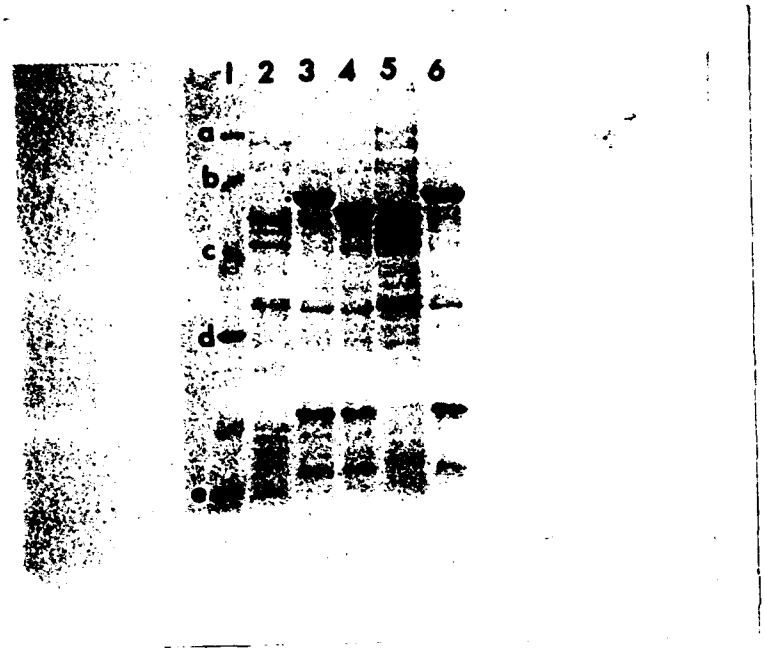



*Figure 9: Expression of rhAChE variants: 50ml cultures of indicated clones were grown to $OD_{660}$ of 3.5 in LB. Inclusion bodies were isolated and partially purified by two successive washes in water. The inclusion bodies were solubilized in 1ml of 8.0M urea (each) and 15µl were subjected to SDS-PAGE on 10% acrylamide gel. Lane 1: M.W. markers of 94, 67, 43, 30 and 20kD (a, b, c, d, and e, respectively); lane 2: Sϕ930 control culture harboring no plasmid; lane 3: Sϕ930pMFL-52Ser; lane 4: Sϕ930pMFEG-8; lane 5: Sϕ930pMFEG-89; lane 6: Sϕ930pMFE-5234. The protein bands marked by dots correspond to the rhAChE. The gel was stained with Coomassie Brilliant Blue.*

TABLE 3

REFOLDING EFFICIENCY OF DIFFERENT rhAChE MUTANTS

| CLONE | TYPE OF CHAIN | ACTIVITY (U/ml) | PROTEIN (mg/ml) | SPECIFIC ACTIVITY |
|---|---|---|---|---|
| Sϕ930pMFEG-8 | truncated (C-terminus) | 5.84 | 0.099 | 59.0 |
| | | 3.60 | 0.077 | 46.8 |
| | | 4.24 | 0.072 | 58.7 |
| | | 3.97 | 0.088 | 46.3 |
| Sϕ930pMFL-52Ser | nontruncated (control) | 1.40 | 0.071 | 19.8 |
| | | 1.09 | 0.055 | 20.0 |
| | | 1.68 | 0.088 | 19.0 |
| | | 1.04 | 0.064 | 16.2 |
| Sϕ930pMFE-5234 | substitute of $Cys^{257}$ and $Cys^{272}$ to Ser | 0.001 | 0.080 | 0.01 |
| Sϕ930pMFEG-89 | truncated C-terminus, N-terminus | 0 | 0.082 | 0 |

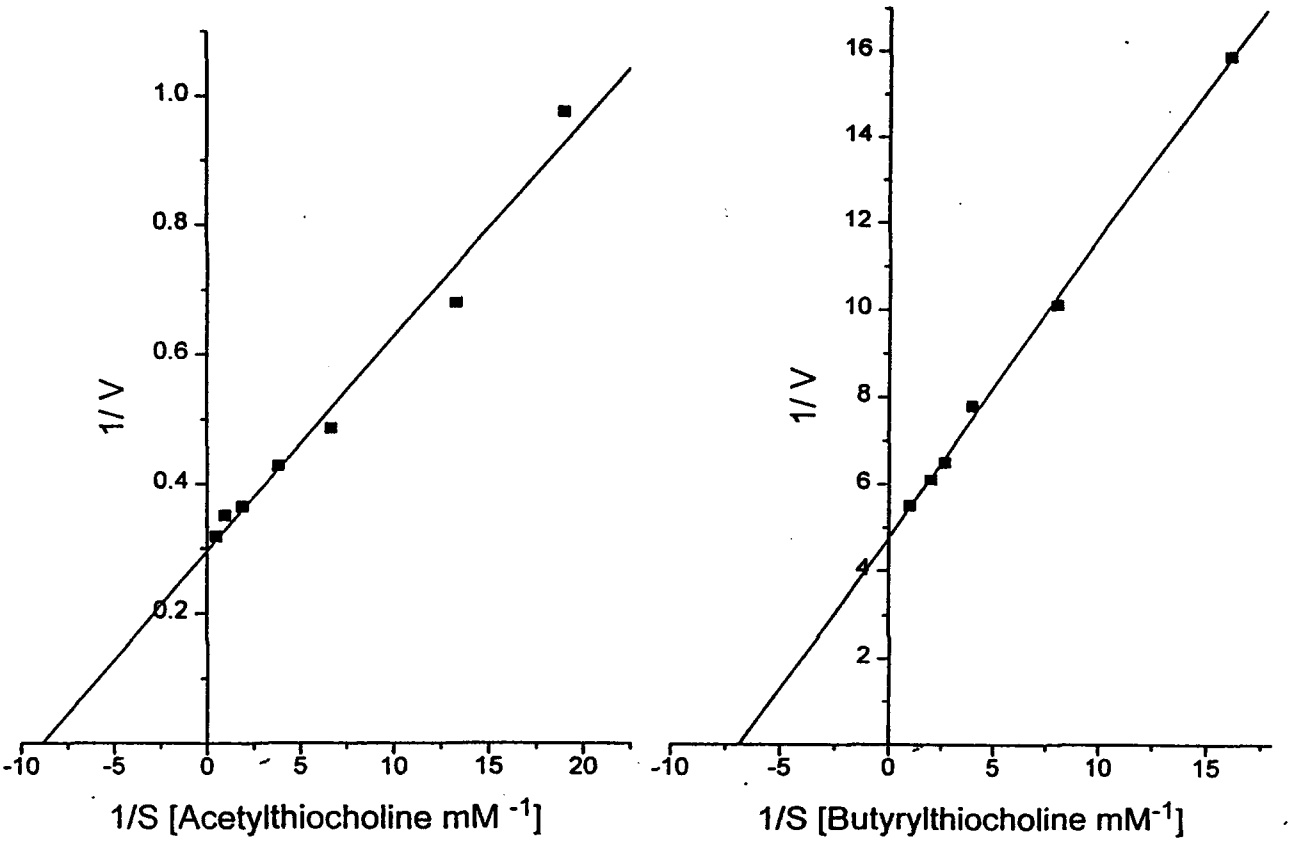



*Figure 10: Lineweaver–Burk plots for $K_m$ determinations with acetylthiocholine and butyrylthiocholine of refolded truncated nonpurified rhAChE derived from Sϕ930pMFEG–8.*

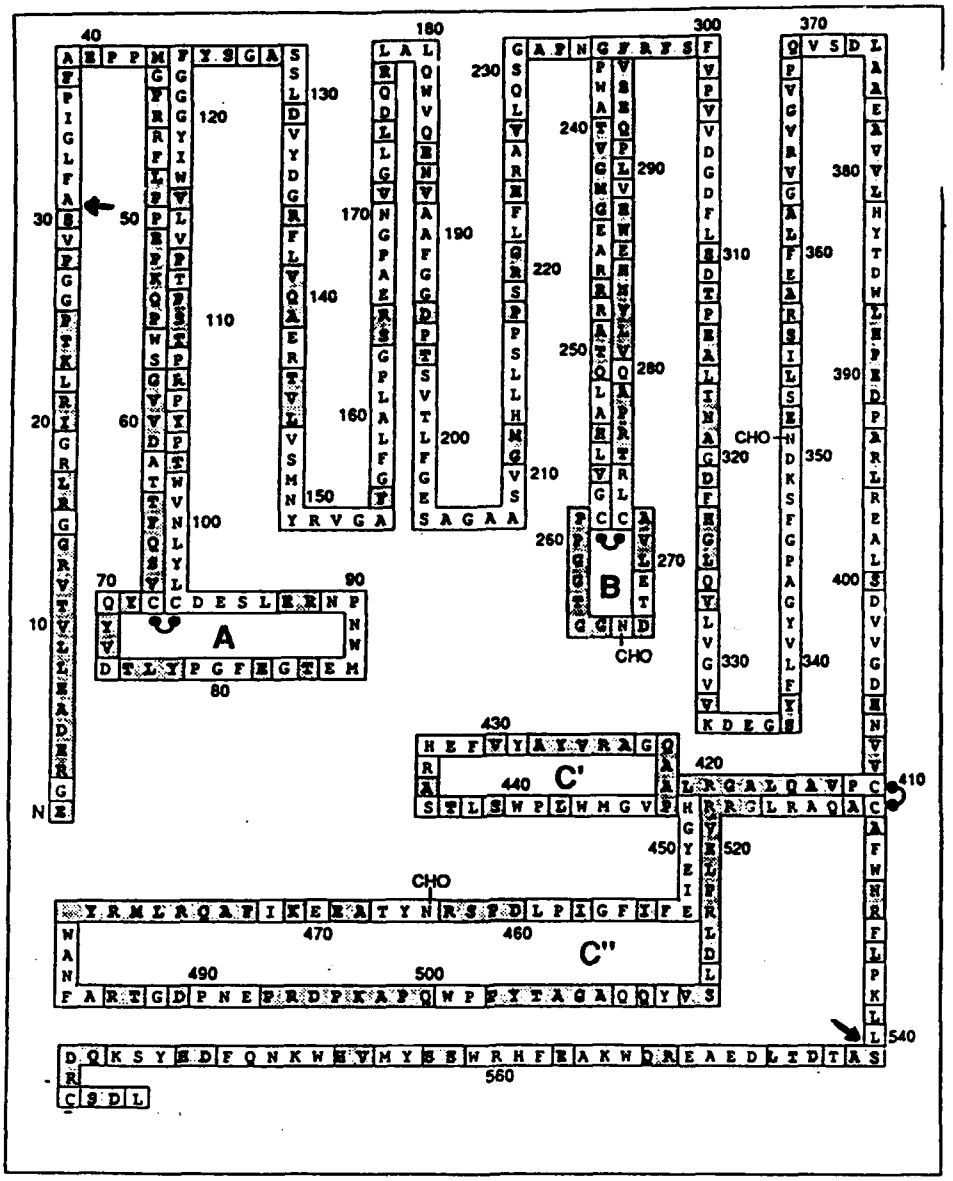



*Figure 11: The two-dimensional scheme of rhAChE amino acid sequence based on the model for human BuChE presented by Neville et al. (1992). Amino acid residues, in the single letter code, are numbered starting with the N-terminus. Disulfide bonds are dotted and interconnected. The cysteine loops A, B, C and C" are marked. The arrow at amino acid 540 denotes the last amino acid (lue) in the truncated rhAChE specified by clone Sϕ930pMFEG-8. Sϕ930pMFEG-89 expresses the amino acid sequence between residue 30 and 540, as indicated by both arrows.*

## 3.2 Manipulation of refolding conditions to improve yields

Table 4 summarizes the results of experiments aimed at improving the refolding yield of rhAChE. The second column in the table lists the additives present in the rhAChE solubilization solution in addition to 8.5M urea. The average MW of PEG and its concentration in refolding solutions are shown in the third and fourth columns, respectively. The last column is the calculated specific activity (S.A) of the refolded enzymatically active rhAChE. The results of these manipulations did not yield a significant improvement of AChE recovery. The minor changes in the S.A of the refolded rhAChE could be due to the experimental fluctuations.

Results of rhAChE reconstitution into enzymatically active form in the presence of a new detergent 6-0-(N-heptylcarbamoyl)-methyl-α-D-glucopyranoside (HECAMEG, produced by VEGATEC Co. France), that has a high critical micellar concentration of 19.5mM (6.5g/L) and is able to reduce nonspecific hydrophobic interaction, did not differ from those obtained under other conditions.

To determine the effect of [protein]/[PEG] on reconstitution of rhAChE, the 8.5M urea containing the solubilized inclusion bodies was diluted into refolding solution to yield the following ratios of [Protein]/[PEG]: 1:10, 1:20, 1:40 1:100, 1:200 and 1:850 (mol/mol). The refolding solution in a final volume of 10ml contained 1.2-1.5mg protein which is equivalent to 18-23nmol of rhAChE. For example, the [Protein]/[PEG] ratio of 1:20 contained 23nmol rhAChE and 460nmol PEG. The refolding was carried out for 48h at 4°C. One ml of each sample was dialyzed against 1L of 10mM L-arginine pH 9.4-10.0 containing 0.3mM of GSSG for 16-18h. AChE activity was determined after 48h incubation of the dialyzed material at room temperature.

TABLE 4

*IN VITRO* REFOLDING OF rhAChE: EFFECT OF PEG AND TMAC

| # | Dilution (1:20) into 8.5M urea containing | Dilution (1:10) into refolding buffer containing | | S.A. |
|---|---|---|---|---|
| 1 | no additives (control) | PEG-3450 | 0.3% | 25 |
| 2 | - " - | PEG-3450 | 0.6% | 18 |
| 3 | - " - | PEG-2000 | 0.3% | 22 |
| 4 | - " - | PEG-2000 | 0.6% | 21 |
| 5 | - " - | PEG-1000 | 0.3% | 23 |
| 6 | 0.1M TMAC | PEG-3450 | 0.3% | 24 |
| 7 | PEG-3450 (0.3%) | PEG-3450 | 0.3% | 20 |
| 8 | PEG-3450 (0.3%) and 0.1M TMAC | PEG-3450 | 0.3% | 20 |
| 9 | | PEG-3450 1mM HECAMEG | 0.3% | 24 |
| 10 | no additives | no PEG | - | 12 |

*Enzyme activity was determined 48h after incubation at room temperature. Protein was determined according to Bradford (1976) and the specific activity (S.A.) expressed as U/mg protein/min at 25°C.*

Table 5 summarizes the results of experiments aimed to test the effect of PEG on refolding at various [protein]/[PEG] ratios. The specific activities of the refolded enzymatically active rhAChE show a minor difference. Similar manipulations of refolding conditions for carbonic anhydrase, rhDNase and recombinant tPA resulted in considerable improvement (Cleland and Wang, 1990; Cleland et al., 1992).

TABLE 5

EFFECT OF rhAChE/PEG (mol/mol) ON REFOLDING

| PROTEIN/ PEG | rhAChE (nmol protein/10ml) | PEG/10ml (nmol) | U/ml | SPECIFIC ACTIVITY |
|---|---|---|---|---|
| 1:10 | 23 | 230 | 3.6 | 24 |
| 1:20 | 23 | 460 | 3.2 | 22 |
| 1:40 | 18 | 720 | 3.3 | 28 |
| 1:100 | 20 | 2000 | 2.6 | 20 |
| 1:200 | 21 | 4200 | 2.7 | 20 |
| 1:850 (control) | 18 | 15300 | 2.6 | 24 |

*Protein concentration of the solubilized rhAChE was 120–150μg/ml which is equivalent to 18–23 nmol of rhAChE. The molecular weights of PEG and rhAChE used for calculations were 3450 and 62kD, respectively. Specific activity (S.A.) was calculated as U/mg protein/min at 25°C.*

## 3.3 Purification of rhAChE

3.3.1 Small scale partial purification of rhAChE: Since higher AChE activity was obtained after refolding with the mutant enzyme harboring the $Cys^{580} \rightarrow Ser^{580}$ substitution, we have focused on the purification of this analogue. One liter of reconstituted rhAChE containing 1260 units was concentrated and dialyzed using a "Pellicon" dialysis concentrator and a 30kD MW cutoff membrane. The volume was reduced to 600ml in 20mM HEPES, pH 8.0 and applied to a Q–Sepharose column chromatography. Table 6 summarizes the results of purification. Pooled fractions, containing AChE activity, were eluted from Q–Sepharose at about 0.275–0.375mM NaCl. Calculations of yield showed that 64% of the activity was recovered with a specific activity of 117U/mg. After precipitation with 45% saturation of ammonium sulfate and dialysis against 20mM HEPES, pH 8.0, 180 units were applied to MAC–Sepharose 4–B affinity column (1ml bed volume). As shown in Table 6, the affinity chromatography step improved purity by 19–fold, with an overall recovery of 84%. SDS–PAGE analysis of purified rhAChE revealed a single protein band on Coomassie Brilliant Blue stained acrylamide gel (not shown).

TABLE 6

SMALL SCALE PURIFICATION OF rhAChE

| | VOLUME (ml) | ACTIVITY (units) | PROTEIN (mg) | SPECIFIC ACTIVITY (U/mg protein) |
|---|---|---|---|---|
| Q–Sepharose load | 600 | 1260 | 30 | 42 |
| Q–Sepharose eluate | 23 | 805 | 7.8 | 117 |
| MAC–Sepharose load | 1 | 180 | 1.5 | 120 |
| MAC–Sepharose eluate | 10 | 151 | 0.066 | 2289 |

*Inclusion bodies derived from Sϕ930pMFL–52Ser were solubilized and subjected to in vitro refolding. Following concentration–dialysis, rhAChE was first subjected to Q–Sepharose ion–exchange chromatography. The pool of active fractions, eluted by NaCl gradient, was processed and subjected to MAC–Sepharose 4B affinity chromatography. Note that only 180 out of 805 units were loaded onto MAC–Sepharose affinity column due to the limiting capacity of the small bed volume. After repetition of the affinity step several times to process the entire batch of 805 units, active fractions were pooled, concentrated by ammonium sulphate precipitation (45% saturation), resuspended in 1ml of 20 mM HEPES, pH 8.0, 2.5mM EDTA and dialyzed against 4L of the same buffer. A total of 0.290mg protein containing 661 units of active rhAChE was recovered (52.5% yield by activity).*

3.3.2 Pilot–scale purification of rhAChE: Tables 7 to 9 summarize the data on rhAChE refolding and purification of three batches. For example, solubilization of purified inclusion bodies yielded 70.4g of total protein (Table 9). The level of contaminating proteins in the preparation that amount up to 30% was determined by densitometric analysis of SDS–PAGE (not shown). Thus, 49.2g of denatured rhAChE yielded $1.9 \times 10^6$ units of active enzyme prior to purification. This represents 415mg of enzyme, assuming that the highest attainable specific activity is 4572 U/mg. Refolding of rhAChE into an enzymatically active form after removal of salts occurred in a time–dependent manner, as shown in Figure 12, with a half–life of approximately 20h. Although the efficiency of refolding is low (close to 1%), the yield of active enzyme was 66% of the initial activity. Most of the inactive rhAChE that did not bind to DEAE–Sepharose was soluble aggregated material as determined by Sephacryl S400 gel filtration (Figure 13). Attempts to reduce aggregation by incorporation of nonionic detergents such as Nonidet P40 or Triton X–100 were ineffective. The 4.14g of protein eluted from the DEAE

column contained about 87% of inactive rhAChE that did not bind to the MAC affinity column. This inactive rhAChE that was further purified by Q-Sepharose chromatography contained a homogenous dimeric form as determined by FPLC on Superose-12 (data not shown). The electrophoretic pattern of the purified protein is shown in Figure 14.

The subunit character of the mutant rhAChE was determined by gel permeation chromatography on Sephacryl 300. The column was equilibrated with 10mM L-arginine, pH 10.0, and 50 mM NaCl. Bovine serum albumin containing the 67kD monomer and the 135kD dimer was used as an internal control. About 50 units of the highly purified recombinant enzyme was mixed with BSA and applied to the column. Figure 15 shows that the 135kD BSA dimer eluted at fraction 45 and the 67kD monomer eluted at fraction 50, while the activity of the rhAChE peaked in fraction 53. Similar analysis by HPLC with a Superose 12 column consistently resulted in elution of the 67kD BSA monomer prior to the elution of active rhAChE (data not shown). These results indicate that the *in vitro* reconstituted active rhAChE is a monomer.

TABLE 7

SUMMARY OF RECONSTITUTION AND PURIFICATION OF rhAChE DERIVED FROM *E. COLI* Sϕ930pMFL-52Ser – Run #1

| STEP | ACTIVITY (U/ml) | PROTEIN (mg/ml) | TOTAL VOLUME (ml) | TOTAL UNITS | TOTAL PROTEIN (mg) | SPECIFIC ACTIVITY (U/mg) | RE-COVERY BY ACTIVITY (%) |
|---|---|---|---|---|---|---|---|
| *Solubilized I.B. in GTC | – | 11 | 125 | – | 1375 | – | – |
| Dilution (1:25) into 8.5M urea | – | 0.55 | 2500 | – | 1375 | – | – |
| Dilution (1:10) into refolding buffer | – | 0.04 | 30000 | – | 1330 | – | – |
| After con-centration/ dialysis on 10K membrane | 69.2 | 1.32 | 960 | 66240 | 1266 | 52.3 | 100 |
| DEAE column chroma-tography | 1910 | 1.53 | 32 | 61120 | 49 | 1248 | 92 |
| **MAC affinity and analysis | 1610 | 0.33 | 25.1 | 40412 | 8.37 | 4869 | 61 |

**21g of cell dry weight (150g wet weight) was used as starting material*
***Fractions analyzed by SDS-PAGE stained with Coomassie Blue and containing no apparent contaminating protein bands were pooled. Assuming specific activity of 6000 for purified rhAChE, then 66240 units represent 11mg of refolded enzyme. The efficiency of refolding is thus 0.87%.*

TABLE 8

SUMMARY OF RECONSTITUTION AND PURIFICATION OF rhAChE DERIVED FROM *E. COLI* Sϕ930pMFL-52Ser - Run #2

| STEP | ACTIVITY (U/ml) | PROTEIN (mg/ml) | TOTAL VOLUME (L) | TOTAL UNITS $x10^6$ | TOTAL PROTEIN (g) | SPECIFIC ACTIVITY (U/mg) | RE-COVERY BY ACTIVITY (%) |
|---|---|---|---|---|---|---|---|
| *Solubilized I.B. in GTC | - | 19.30 | 1.5 | - | 29.0 | - | - |
| Dilution (1:20) into 8.5M urea | - | 0.72 | 40.0 | - | 28.8 | - | - |
| Dilution (1:10) into refolding buffer | - | 0.07 | 405.0 | - | 28.7 | - | - |
| After con-centration/ dialysis on 10K membrane | 17.9 | 0.20 | 95.0 | 1.70 | 19.0 | 89 | 100 |
| DEAE column chroma-tography (pooled fractions) | 240 | 0.25 | 7.3 | 1.75 | 1.856 | 941 | 100 |
| **MAC | 4927 | 0.83 | 0.2 | 1.01 | 0.169 | 5936 | 59.4 |

**560g of cell dry weight (4000g wet weight) was used as starting material*

***Fractions analyzed by SDS-PAGE stained with Coomassie Blue and containing no apparent contaminating protein bands were pooled. Assuming specific activity of 6000 for purified rhAChE, then (prior to purification) $1.7x10^6$ units represent 283mg of refolded enzyme. The efficiency of refolding is thus 1.49% (283/19000).*

## TABLE 9

## SUMMARY OF RECONSTITUTION AND PURIFICATION OF rhAChE DERIVED FROM *E. COLI* SΦ930pMFL-52Ser - Run #3

| STEP | ACTIVITY (U/ml) | PROTEIN (mg/ml) | TOTAL VOLUME (L) | TOTAL UNITS x10$^6$ | TOTAL PROTEIN (g) | SPECIFIC ACTIVITY (U/mg) | RE-COVERY BY ACTIVITY (%) |
|---|---|---|---|---|---|---|---|
| Solubilized I.B. in GTC | - | 32 | 2.2 | - | 70.4 | - | - |
| Dilution (1:23) into 8.5M urea | - | 1.40 | 50 | - | 70.0 | - | - |
| Dilution (1:10) into refolding buffer | - | 0.11 | 600 | - | 66.0 | - | - |
| After concentration/ dialysis on 10K membrane | 14.6 | 0.50 | 130 | 1.90 | 65.0 | 29.2 | 100 |
| DEAE column chroma-tography | 170 | 0.46 | 9 | 1.53 | 4.14 | 370 | 80 |
| MAC affinity chroma-tography #1 | 20649 | 7.42 | 0.072 | 1.49 | 0.534 | 2759 | 78 |
| MAC affinity chroma-tography #2 | 42059 | 9.30 | 0.030 | 1.26 | 0.281 | 4572 | 66 |

*MAC-Sepharose affinity chromatography was repeated three times in order to process the entire batch and then pooled. The pooled fractions were precipitated with 35% saturated ammonium sulfate and then dialyzed against 20mM HEPES pH 8.0, containing 2.5mM EDTA. The activity and protein concentration after dialysis are presented in Table 9 as MAC affinity chromatography #1. The dialyzed pool of MAC affinity chromatography No. 1 was reapplied to the newly equilibrated affinity column and eluted with 0.2M L-arginine pH 10.0. Fractions were pooled and concentrated to a final volume of 30ml in an Amicon diafiltration unit equipped with a 10K membrane. The concentrated material was then dialyzed against 20mM HEPES pH 8.0, containing 50mM NaCl and 2.5mM EDTA. The enzyme activity and protein concentration are presented in Table 9 as MAC-Affinity #2.*

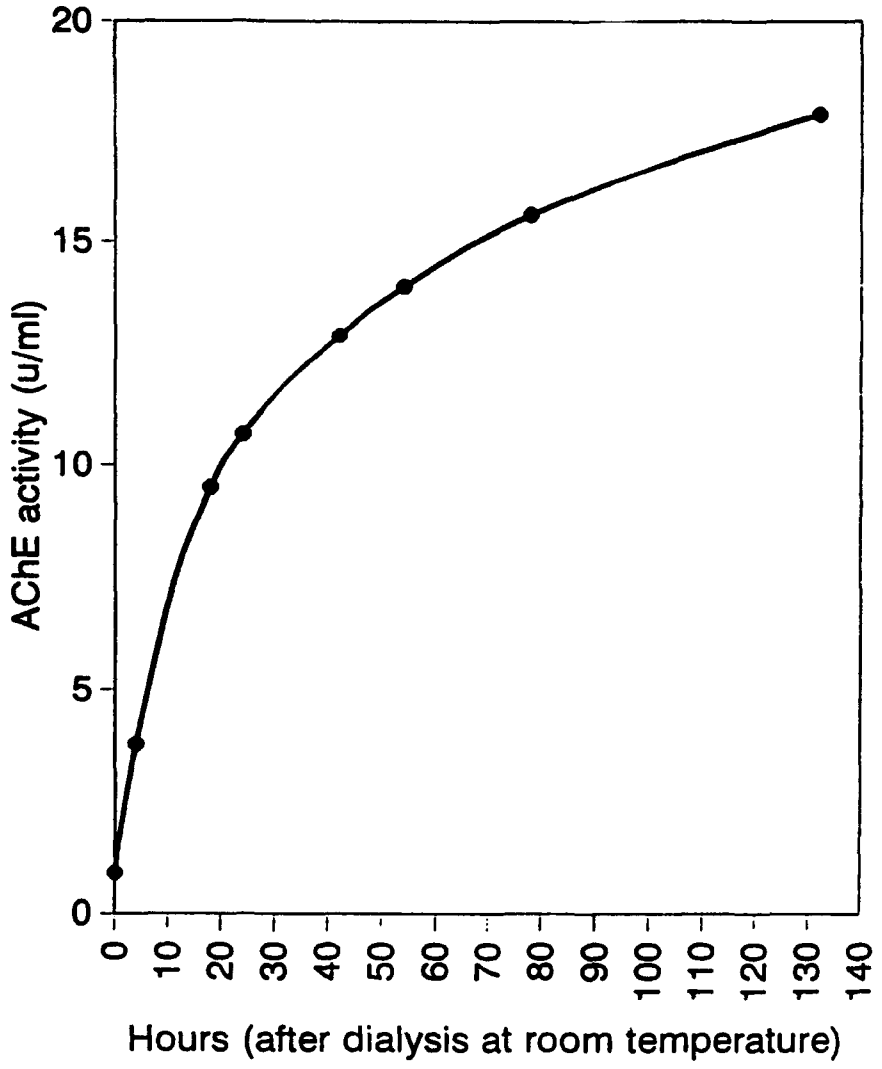



*Figure 12: Renaturation kinetics of rhAChE*

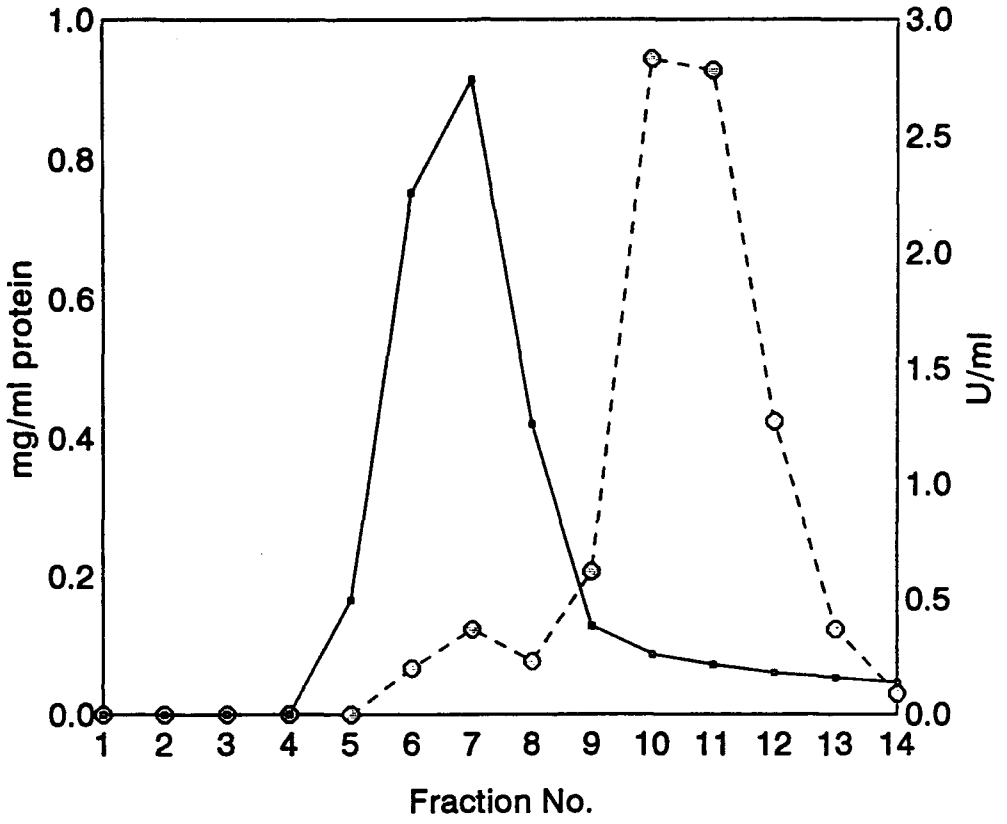



*Figure 13: Sephacryl–S400 size exclusion chromatography. 7ml of DEAE–Sepharose wash through material containing about 25mg of inactive rhAChE was seeded with about 100 units of purified rhAChE (25µl of enzyme with a specific activity of 4572) and applied to the column equilibrated with 20ml HEPES, pH 8.0, 2.5mM EDTA. Fractions of 5ml were collected at a rate of 30ml/h. The column (16x630mm) contained 140ml of resin. The void volume was 35ml (fraction #7). Dotted line: rhAChE activity; solid line: protein concentration.*


*Figure 14: SDS–PAGE pattern of purified rhAChE on 10% acrylamide. Lane 1 – 8μg of purified enzyme; lane 2 – M.W. markers. From top to bottom 97, 66, 43 and 30kD.*

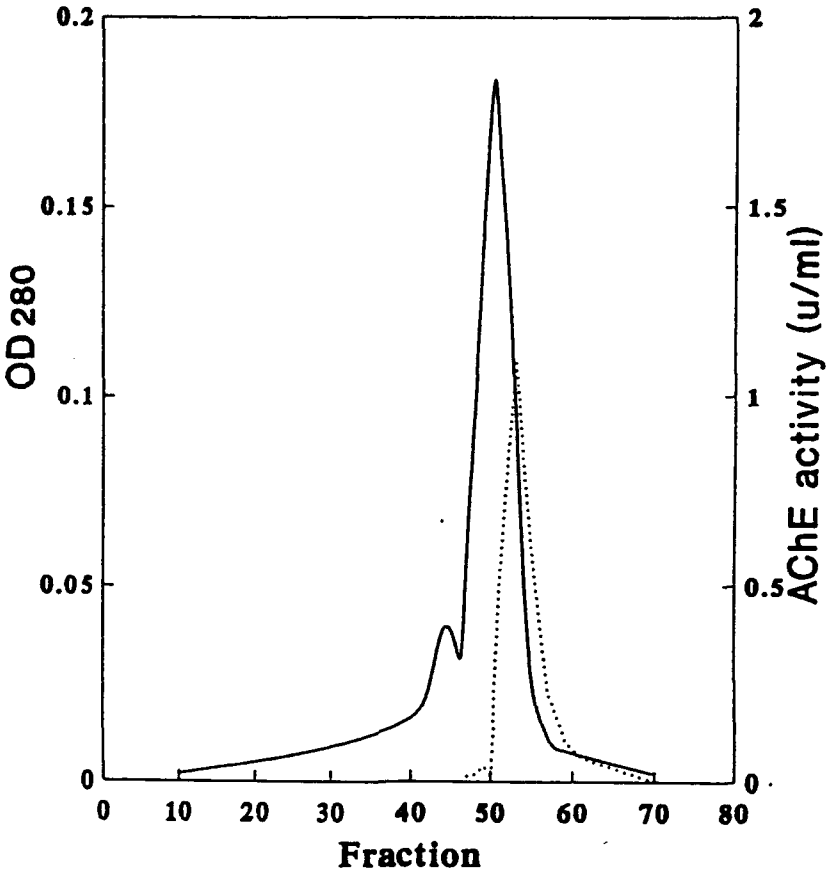



*Figure 15: Molecular weight determination of active rhAChE. Purified rhAChE obtained from clone Sϕ930pMFL-52Ser was mixed with bovine serum albumin (BSA) and subjected to size exclusion chromatography on Sephacryl-300. Absorbance was monitored at 280nm, and AChE activity was determined in each fraction. The solid line shows the elution pattern of BSA containing a minor peak corresponding to a M.W. of 135kD of dimers and the larger peak corresponds to BSA monomer of 67kD. The dotted lines show an elution profile of rhAChE as determined by activity. A total of 8mg of BSA containing 50 units of rhAChE in 18ml was applied to a column of 720x26mm (Pharmacia).*

## 3.4 Biochemical characterization of rhAChE

3.4.1 Catalytic properties of purified rhAChE: $K_m$ values for acetylthiocholine and butyrylthiocholine for the $Ser^{580}$ mutant rhAChE were calculated by the method of Lineweaver and Burk (Figure 16). The apparent $K_m$ obtained with acetylthiocholine was 0.1158 ± 0.0067mM (three independent determinations), while with butyrylthiocholine the $K_m$ was 12.5 ± 0.7 mM. The $K_m$ of the crude recombinant AChE, derived from clone Sϕ930pAIF-52 harboring the native amino acid $Cys^{580}$, was 0.125mM for acetylthiocholine (Figure 17) and indicates that the substitution of $Cys^{580}$ to Ser did not alter the catalytic properties.

To define further the properties of the mutant rhAChE, its inhibition by the AChE specific inhibitor BW284C51 and the BuChE specific inhibitor iso-OMPA were compared (Figure 18). The sigmoid profiles of inhibition obtained for the purified enzyme and the native erythrocyte AChE are quite similar. 50% inhibition was obtained at 1nM of BW284C51 for both enzymes. A 50% inhibition with iso-OMPA was obtained at 0.9mM, which is six orders of magnitude higher relative to the inhibitory concentration of BW284C51, and is comparable with results obtained for rhAChE expressed in cell culture (Velan et al., 1991a).

3.4.2 Stability of purified rhAChE: The purified rhAChE was diluted into buffer devoid of BSA to a concentration of 0.6-1μg/ml, that is equivalent to 2-3U/ml, and lost 90% of its activity within 3h of incubation at room temperature (Figure 19), whereas inactivation was not observed in the presence of 0.5mg/ml BSA. Incubation at 37°C, however, resulted in a 20% loss of activity within the first hour with no additional loss thereafter. rhAChE kept at 4°C as a 5-14mg/ml stock solution was found to be fully stable for at least 6 months (data not shown; assays were performed following dilution into buffer containing BSA).

3.4.3 pH stability profile: The comparison of the pH stability profiles for the human erythrocytes and rhAChE presented in Figure 20 suggests that the recombinant enzyme is more susceptible to inactivation in the pH range of 4.0 - 7.0. Full activity was retained after 10 days of incubation at pH 9.0, for the recombinant enzyme, while the human erythrocyte-derived enzyme retained full activity at both pH 8.0 and 9.0. Both enzymes are equally susceptible to inactivation in the pH range of 10.0 - 11.0.

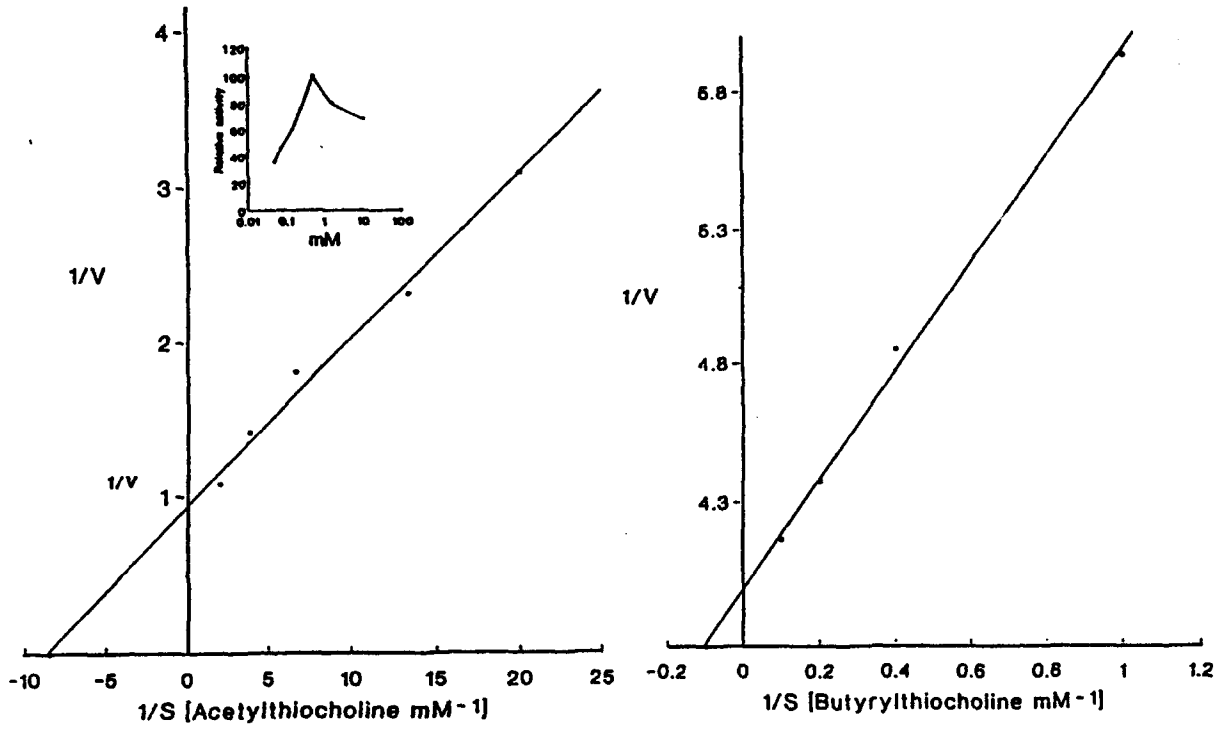



*Figure 16: Kinetic parameters of purified rhAChE reconstituted in vitro: double-reciprocal Lineweaver and Burk plot and substrate inhibition (inset). $K_m$ was determined from the Lineweaver and Burk plot. Constant volume of highly purified rhAChE derived from clone Sϕ930pMFL-52Ser was added to the assay mix containing the indicated concentrations of acetylthiocholine or butyrylthiocholine. Activity was monitored in a PU8700 spectrophotometer.*

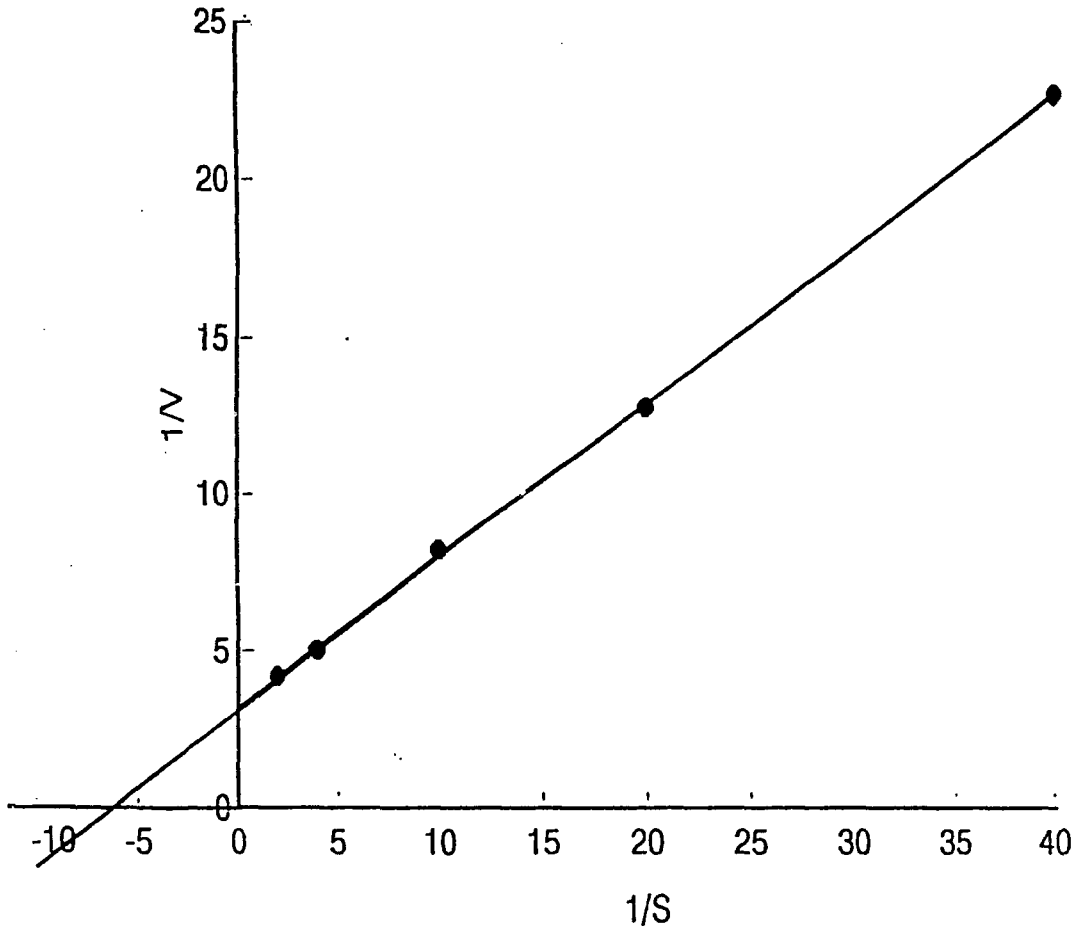



*Figure 17: $K_m$ determination of rhAChE derived from clone Sϕ930pAIF−51 which harbors the native amino acid sequence of hAChE catalytic subunit. Crude nonpurified active enzyme obtained after in vitro refolding was used for this determination.*

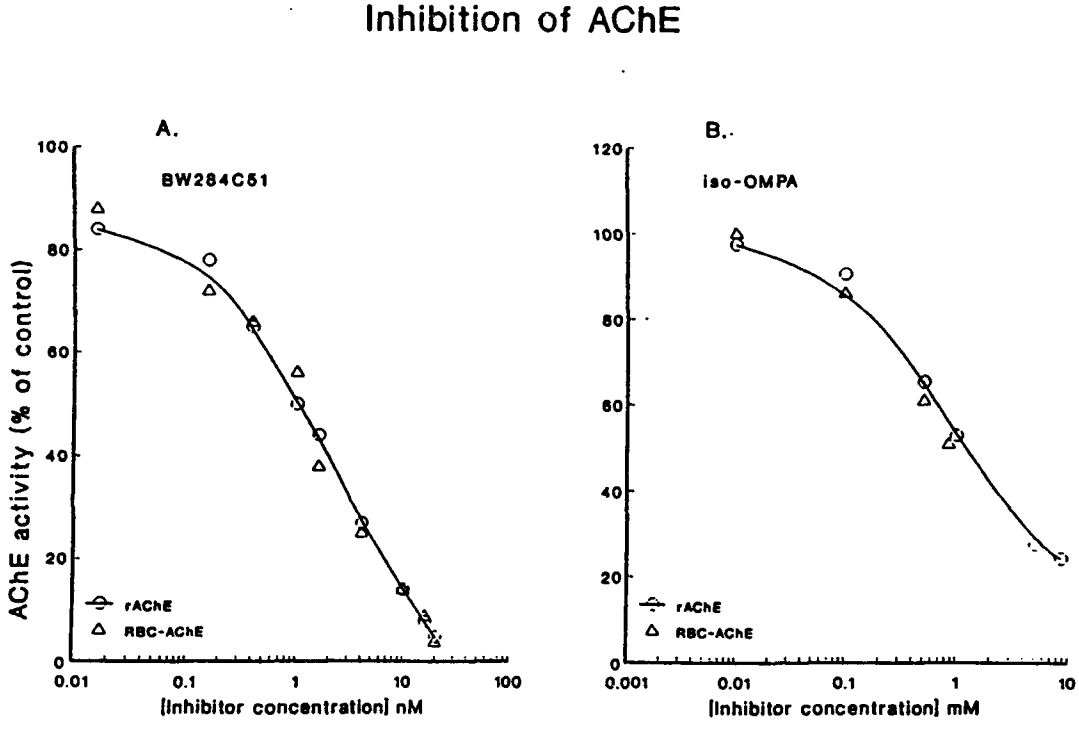



*Figure 18: Effect of inhibitors on rhAChE. Highly purified rhAChE (o) derived from clone Sϕ930pMFL−52Ser and erythrocyte AChE (Δ) obtained from Sigma Co. (St. Louis) were assayed in the presence of BW284C51 (panel A) or iso−OMPA (panel B) at indicated concentrations.*

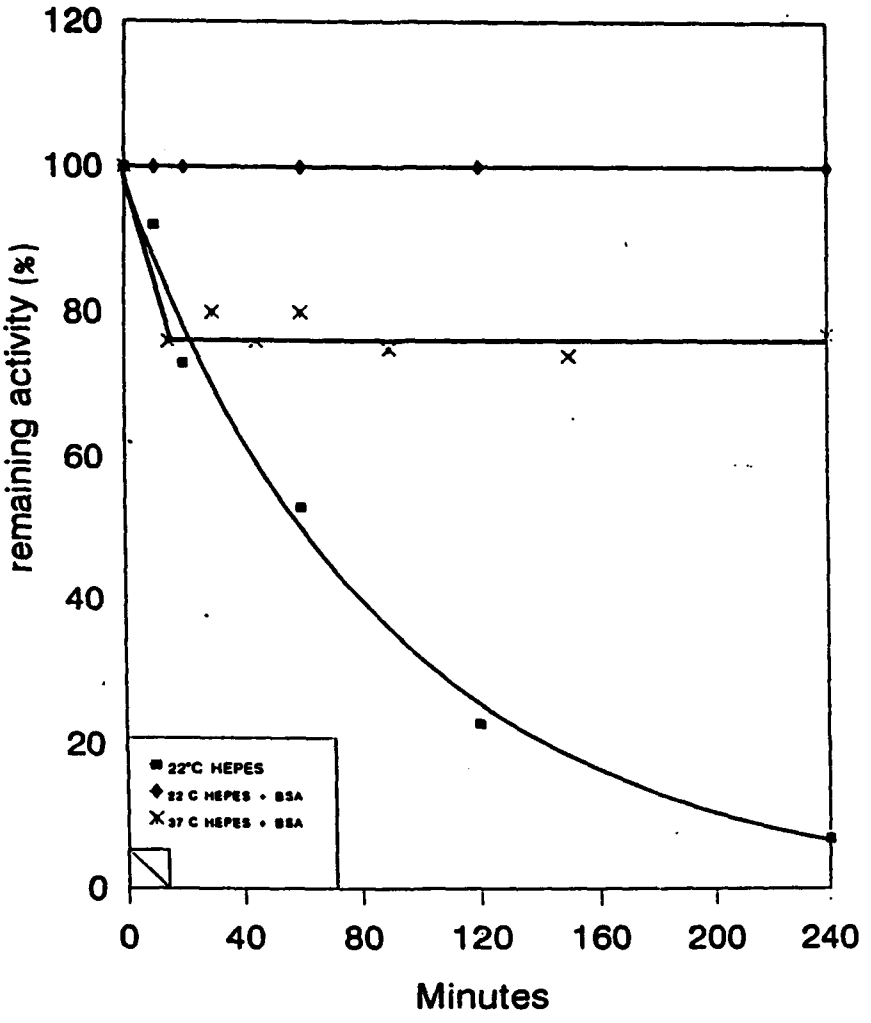



*Figure 19: Stability of rhAChE at 22 and 37°C. Purified rhAChE ($Cys^{580}$→$Ser^{580}$) was diluted into 0.1M HEPES pH 8.0, with and without 0.5 mg/ml BSA to yield a final concentration of 2-3 units/ml. The diluted enzyme was incubated at indicated temperatures, and at time intervals samples were removed for determination of activity. The observed activity immediately after dilution was used as reference point for full activity.*

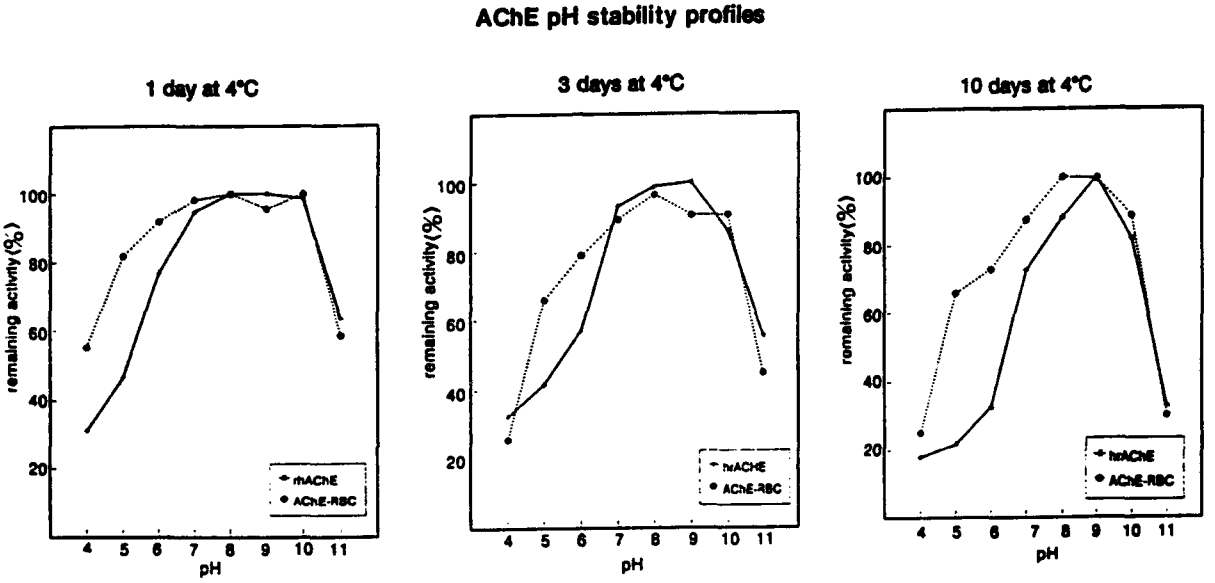
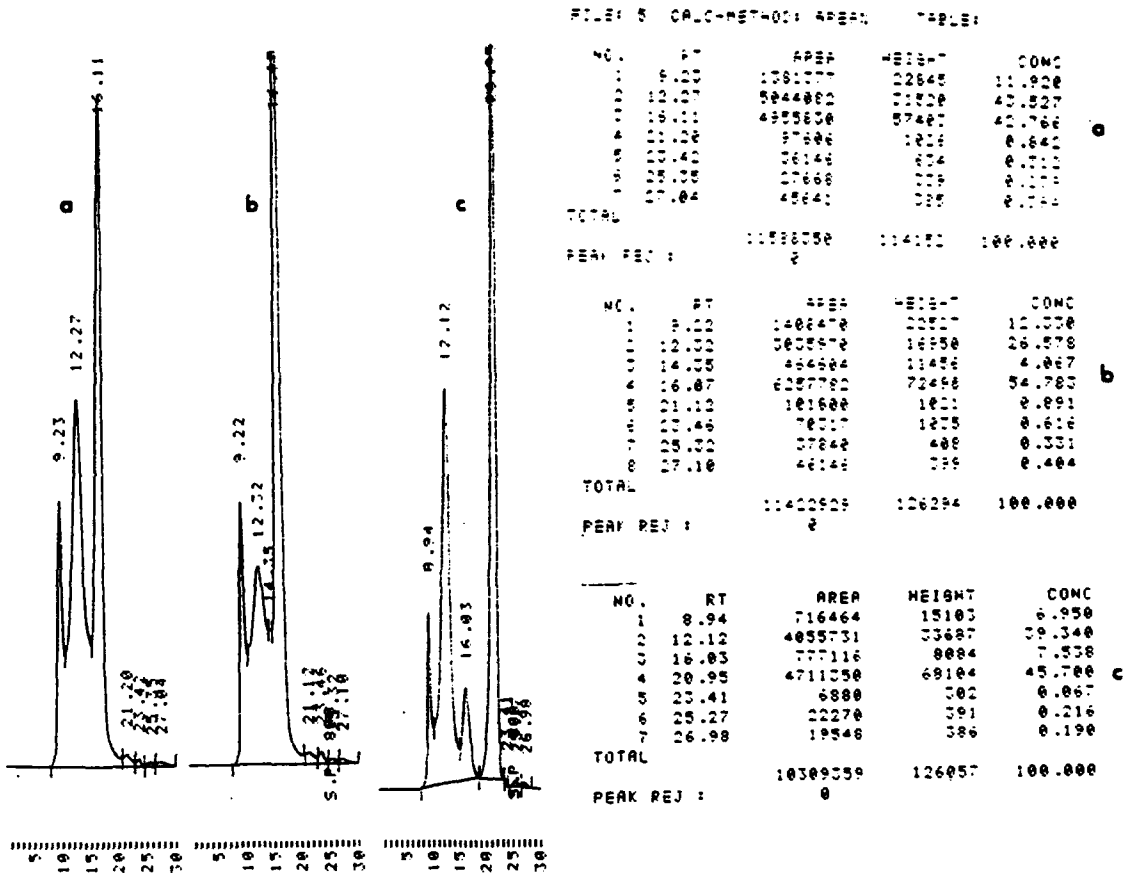



*Figure 20: pH stability of rhAChE ($Cys^{580}$→$Ser^{580}$). The purified rhAChE was diluted into appropriate buffers (see Experimental Procedures) containing 0.5mg/ml BSA to yield a final concentration of 2–3 units/ml. Human erythrocyte AChE (Sigma U.S.A) was dissolved in 0.1M HEPES pH 8.0 containing 0.1% of Triton X–100 and diluted into the same buffers containing 0.5mg/ml BSA to yield 0.6–0.7 units/ml. The diluted enzymes were incubated at 4–5°C for a period of 10 days and activity was determined daily. Panels A, B and C show the pH stability profiles at day 1, 3 and 10 respectively. AChE–RBS denotes human erythrocyte AChE.*

3.4.4 FPLC analysis of purified rhAChE: Purified rhAChE at a concentration of 8–12mg/ml in 20mM HEPES, pH 8.0 and 50mM NaCl, was divided into three samples; samples (a) and (c) were kept at 4°C for 62 days. After that time period, sample (a) was dialyzed at 4°C against 10mM bicarbonate buffer, pH 9.0. Sample (b) was lyophilized after purification, kept at 4°C for 62 days and then resuspended in $H_2O$ and dialyzed against 10mM carbonate buffer pH 9.0. About 50–100µl of each sample were analyzed on a Superose 12 column. Figure 21 shows the elution pattern of the three samples with the corresponding data analysis output. Samples (a) and (b) are identical, except that Sample (b) was lyophilized. It is apparent that the highly aggregated forms eluted at 9.2 min account for about 12% of the rhAChE. A second aggregated oligomeric form that was smaller in size eluted at about 12.3 min both in samples (a) and (b). However, sample (b), the lyophilized rhAChE, contained 26.5% of these forms, whereas sample (a) contained 43.5%. The peak eluted at about 16 min contained the monomeric form. In sample (b) this monomeric form accounted for nearly 55% of the rhAChE, while sample (a) contained about 43% of monomers. The lower monomeric concentration in sample (a) can be attributed to ongoing slow aggregation of rhAChE kept in solution.

Sample (c) in HEPES, pH 8.0 and 50mM NaCl, kept in solution for the same period of time and analyzed by FPLC, reveals four peaks. The last peak eluted at 20.9 min was not considered AChE, since it eluted on the time scale that corresponds to very low molecular weight peptides and other low molecular weight materials. Thus, in computing the area under the first three peaks, we have recalculated the relative area by excluding the area of the peak eluted at 12.1 min. Therefore, sample (c) contains about 12.8% highly aggregated oligomers that elute at about 9 min, about 72% of smaller aggregates that elute at 16 min and only 13.9% of monomeric forms that elute at 16 min. The high content of intermediate size of aggregated rhAChE could be attributed to either the lower pH of the solution or the composition of the buffer, or both.

The results of this analysis indicate that prolonged incubation of rhAChE in solution at pH 8.0 promotes aggregation which may be reduced by pH manipulation, or perhaps by lyophilization at higher pH values.




| NO. | RT | AREA | HEIGHT | CONC | |
|---|---|---|---|---|---|
| 1 | 9.23 | 1381777 | 22645 | 11.920 | a |
| 2 | 12.27 | 5044882 | 71520 | 43.527 | |
| 3 | 16.11 | 4955630 | 57407 | 42.766 | |
| 4 | 21.20 | 97666 | 1826 | 0.843 | |
| 5 | 23.43 | 36146 | 674 | 0.312 | |
| 6 | 25.35 | 27668 | 529 | 0.239 | |
| 7 | 27.04 | 45641 | 885 | 0.394 | |
| TOTAL | | 11588750 | 154151 | 100.000 | |
| PEAK REJ : | | 2 | | | |

| NO. | RT | AREA | HEIGHT | CONC | |
|---|---|---|---|---|---|
| 1 | 9.22 | 1406479 | 20517 | 12.330 | |
| 2 | 12.32 | 3035979 | 16950 | 26.578 | |
| 3 | 14.25 | 466584 | 11456 | 4.067 | |
| 4 | 16.07 | 6207782 | 72498 | 54.383 | b |
| 5 | 21.12 | 101600 | 1021 | 0.891 | |
| 6 | 23.46 | 70317 | 1275 | 0.616 | |
| 7 | 25.32 | 37842 | 488 | 0.331 | |
| 8 | 27.10 | 46141 | 335 | 0.404 | |
| TOTAL | | 11420525 | 126294 | 100.000 | |
| PEAK REJ : | | 2 | | | |

| NO. | RT | AREA | HEIGHT | CONC | |
|---|---|---|---|---|---|
| 1 | 8.94 | 716464 | 15103 | 6.950 | |
| 2 | 12.12 | 4055731 | 33687 | 39.340 | |
| 3 | 16.03 | 777116 | 8084 | 7.538 | |
| 4 | 20.95 | 4711050 | 69104 | 45.700 | c |
| 5 | 23.41 | 6880 | 302 | 0.067 | |
| 6 | 25.27 | 22270 | 391 | 0.216 | |
| 7 | 26.98 | 19548 | 386 | 0.190 | |
| TOTAL | | 10309059 | 126057 | 100.000 | |
| PEAK REJ : | | 0 | | | |

*Figure 21: FPLC analysis of purified rhAChE. To determine the molecular species of rhAChE present under a number of different conditions, 50–100μg of rhAChE were subjected to FPLC on Superose 12. All samples were kept for 62 days after purification under the conditions described below, prior to analysis.*
*Sample (a): rhAChE kept in 20mM HEPES, pH 8.0, for indicated time, then dialyzed overnight against 10mM carbonate buffer, pH 9.0.*
*Sample (b): Lyophilized after purification, resuspended in $H_2O$ and then dialyzed overnight against 10mM carbonate buffer, pH 9.0.*
*Sample (c): rhAChE maintained in 20mM HEPES 50mM NaCl, pH 8.0.*

3.4.5 Shape of UV spectrum: The UV spectrum of refolded active rhAChE is shown in Figure 22. The distinct shoulder observed at ~290nm is typical of a Trp-rich protein (Donovan et al., 1973) Also typical of a Trp-rich protein is the fact that the absorbance maximum is at 280.4nm, which is closer to the maximum absorbance of Trp than to that of Tyr. The absorbance minimum of the protein was at 250nm, and the ratio between maximum and minimum was 2.52.

3.4.6 Absorption coefficients ($\varepsilon_{1\%}$) of refolded and misfolded rhAChE: The absorption coefficients $\varepsilon_{1\%}$ of refolded active rhAChE was calculated on the basis of absorbance and the protein concentration which was derived from amino acid analysis. The $\varepsilon_{1\%}$ value of 23.1, obtained by this method, compares well with the results obtained on the basis of protein determination (Table 10) by the modified Bradford method (Macart and Gerbaut, 1982), which gave an average value of $\varepsilon_{1\%}$ = 22.2. The $\varepsilon_{1\%}$ for the misfolded inactive rhAChE determined by the same modified Bradford method was 16.2.

3.4.7 Amino acid composition: The amino acid composition of refolded rhAChE was deduced from the amino acid analysis (Table 11). Calculations were based either on the average protein content (method 1) or on the protein content obtained by adding up the mass of all measured amino acids (method 2). Excellent agreement with the theoretical amino acid composition is obtained by both methods for 16 of the 18 measured amino acids (16 Trp and 6 Cys were not recovered, i.e., the total theoretical number of residues was 562). In the case of Ser, extensive loss (~16%) is seen, and it was therefore not taken into account when the average protein content was determined. The value for Leu is also ~10% lower than the theoretical. For all the other amino acids the deviations are approximately in the range of ±6%. Method 1 is more accurate since it does take into account an average protein content.

3.4.8 Isoelectric point of rhAChE: Figure 23A shows the IEF gel after staining with Coomassie Brilliant Blue. It is clear from Figure 23A that the rhAChE does not migrate as a sharp band (lanes 4-7) when compared to the bands of the pI protein markers (lanes 103, 8, 9). Attempts to produce a sharp band by including 6M urea in both the protein sample and the gel did not result in any improvement beyond that shown in Figure 23A. The pI value for rhAChE could not be determined accurately from the pI calibration curve (Figure 23B). However, the data presented indicate that the pI of rhAChE is in the range of 5.5 - 5.8.

3.4.9 Effect of reducing agent on rhAChE migration: Figure 24 shows the effect of reducing agent on the migration of rhAChE in 10% polyacrylamide-SDS gel. The rhAChE protein band in lane 1 was reduced with ß-mercaptoethanol, while the protein band shown in lane 2 was not subjected to reducing agent. The nonreduced protein migrated somewhat more slowly compared to the reduced protein sample. Boiling the sample for 5 min prior to electrophoresis did not affect the mobility of the protein (lanes 4 and 5).

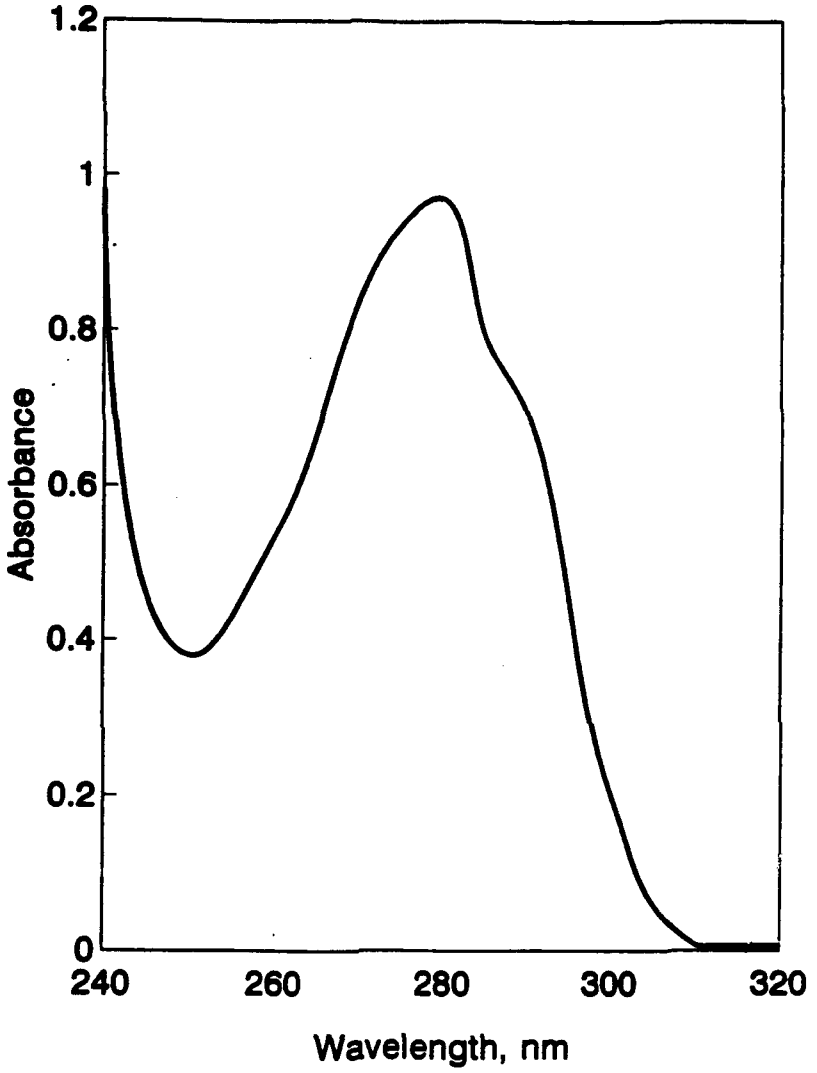



*Figure 22: UV spectrum of purified rhAChE. The spectrum was determined on a sample with a protein concentration of 0.4mg/ml in PBS, pH 7.4. Scanning was obtained on a Phillips scanning spectrophotometer Model PU8720 at a speed of 50 nm/min.*

TABLE 10

EXTINCTION COEFFICIENT OF PURIFIED rhAChE

| SAMPLE | PROTEIN CONCENTRATION (mg/ml) | $A_{280}$[a] | $\varepsilon_{1\%}$ |
|---|---|---|---|
| 1 | 0.40 | 0.966 | 24.1 |
| 2 | 0.12 | 0.274 | 22.8 |
| 3 | 0.415 | 0.819 | 19.7 |
| | | Average | 22.2 |

[a] Corrected for light scattering

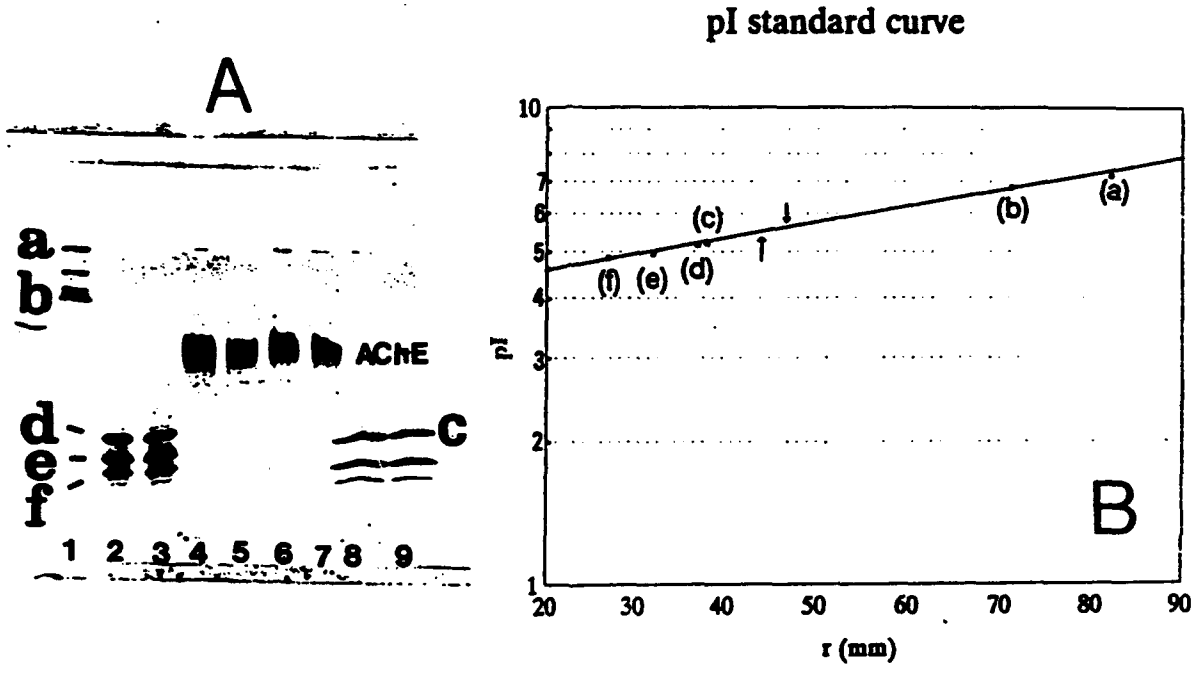



*Figure 23: Isoelectric focusing of rhAChE on polyacrylamide urea gel. A – lane 1: Horse heart myoglobin (Sigma) pI 7.2(a) and 6.8(b); lanes 2 and 3: recombinant human Cu/Zn SOD (BTG) pI 5.16, 4.95(e) and 4.85(f); lanes 4–7: four samples of in vitro refolded and purified rhAChE; lanes 8 and 9: recombinant human growth hormone (BTG), pI 5.2(c). Note: the lower bands in lane 8 and 9 are deamidated forms of rhGH and do not represent the true pI of the protein. The gel was stained with Coomassie Brilliant Blue. B – IEF calibration curve derived from gel shown in A. Arrows indicate the upper and lower boundaries of the rhAChE protein band shown in panel A lanes 4–7. Letters in parentheses correspond to the protein pI markers shown in panel A.*


*Figure 24: Effect of reducing agent on rhAChE electrophoretic mobility. Purified enzyme with and without β–mercaptoethanol was subjected to SDS–PAGE on 10% acrylamide. Lane 1: no reducing agent; lane 2: with reducing agent; lane 3: M.W. markers (94, 67, 43, 30 and 20 kD from top to bottom. Lanes 4 and 5 are the same as in lanes 1 and 2, except that samples in the last 2 lanes were boiled prior to electrophoresis.*

## TABLE 11

## AMINO ACID COMPOSITION OF rhAChE

| AMINO ACID | AMINO ACID CONCENTRATION (nmol) | THEORETICAL RESIDUE NUMBER | OBSERVED RESIDUE NUMBER (METHOD 1[a]) | OBSERVED RESIDUE NUMBER (METHOD 2[b]) |
|---|---|---|---|---|
| Asx | 9.79 | 46 | 46.4 | 46.9 |
| Thr | 5.00 | 25 | 23.7 | 24.0 |
| Ser | 6.19 | 35 | 29.3 | 29.7 |
| Glx | 12.75 | 57 | 60.5 | 61.1 |
| Pro | 9.81 | 47 | 46.5 | 47.0 |
| Gly | 11.66 | 54 | 55.2 | 55.9 |
| Ala | 11.28 | 53 | 53.4 | 54.1 |
| Val | 11.23 | 53 | 53.2 | 53.8 |
| Met | 1.88 | 9 | 8.9 | 9.0 |
| Ile | 2.00 | 9 | 9.5 | 9.6 |
| Leu | 10.95 | 58 | 51.9 | 52.5 |
| Tyr | 4.39 | 21 | 20.8 | 21.0 |
| Phe | 6.14 | 29 | 29.1 | 29.4 |
| His | 2.93 | 14 | 13.9 | 14.0 |
| Lys | 2.16 | 10 | 10.2 | 10.4 |
| Arg | 8.89 | 42 | 42.2 | 42.6 |
| | Total | 562 | 554.7 | 561.0 |

[a]: nmol amino acid/average nmol of protein
[b]: nmol amino acid/Σnmol of 18 amino acids

*The amino acid analysis was performed following hydrolysis of the purified active rhAChE in the gas phase (see experimental section). The data were used to determined the amino acid composition and computation of protein concentration.*

3.4.10 Secondary structure: The overall secondary structures of both refolded and misfolded rhAChE were estimated from the far UV CD spectra shown in Figure 25. The spectrum of refolded rhAChE has a positive peak at 195nm (of around 15000 deg x $cm^2$/dmol) and two negative peaks at 210 and 217nm (around 13000 deg x $cm^2$/dmol). The misfolded inactive rhAChE, on the other hand, displays a broad negative peak centered at 216nm (around 8700 deg x $cm^2$/dmol). From the calculations based on the nonlinear least squares method described under Experimental Procedures, it ensues that the secondary structure of refolded active rhAChE is composed of 39% α-helix 22% ß-sheet and 37% ß-turns and aperiodic structure, whereas that of the misfolded inactive rhAChE is composed of 24% α-helix, 42% ß-sheet and 28% ß-turns and aperiodic structure.

3.4.11 Environment of aromatic residues in rhAChE: Additional structural information can be obtained from the intrinsic fluorescence spectrum. Using excitation at 295nm, where essentially only Trp residues are being excited (Chen et al., 1969), we obtained a fluorescence emission spectrum peaking at 334nm (Figure 26) and 355nm for rhAChE and the Trp model compound N-acetyl-tryptophanamide, respectively. Further information about the Trp environment in the rhAChE was obtained from the determination of the fluorescence efficiency index, $R_{Trp}$ = 1.17, relative to the model compound.

The near UV CD spectrum of refolded active rhAChE (Figure 27) displays a very weak signal, probably due to a reduced degree of helicity in the regions containing tyrosine (to which the 272nm maximum can be assigned) and tryptophan (the 290nm peak).

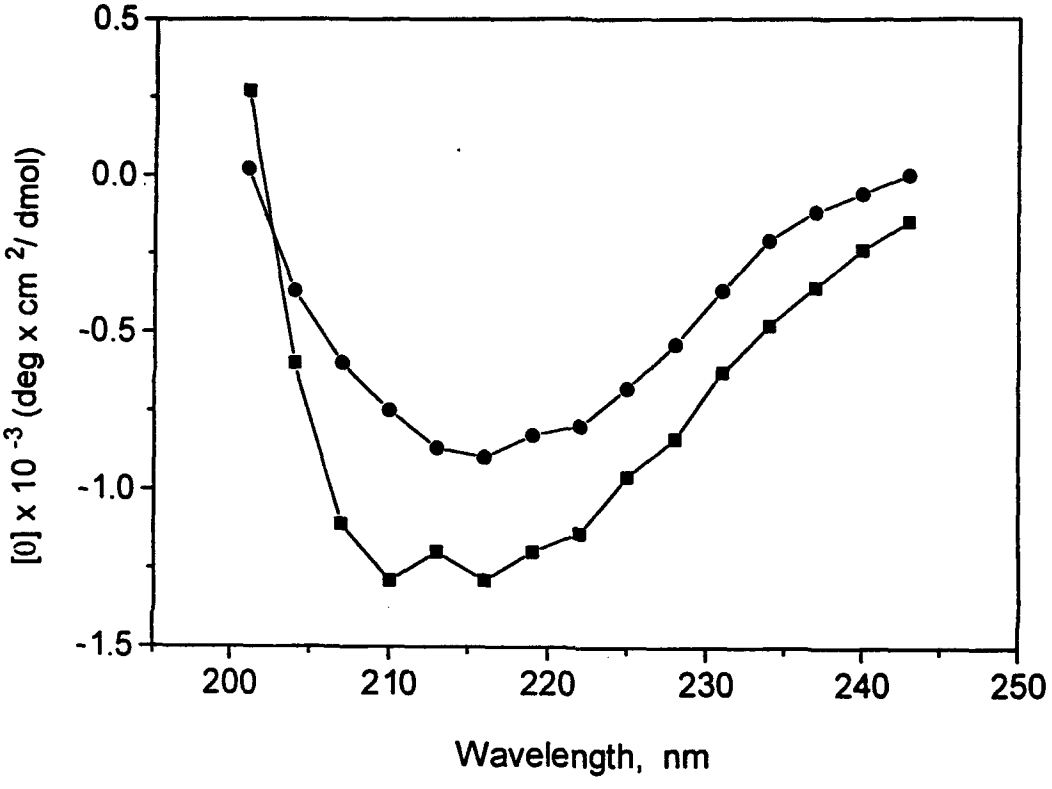
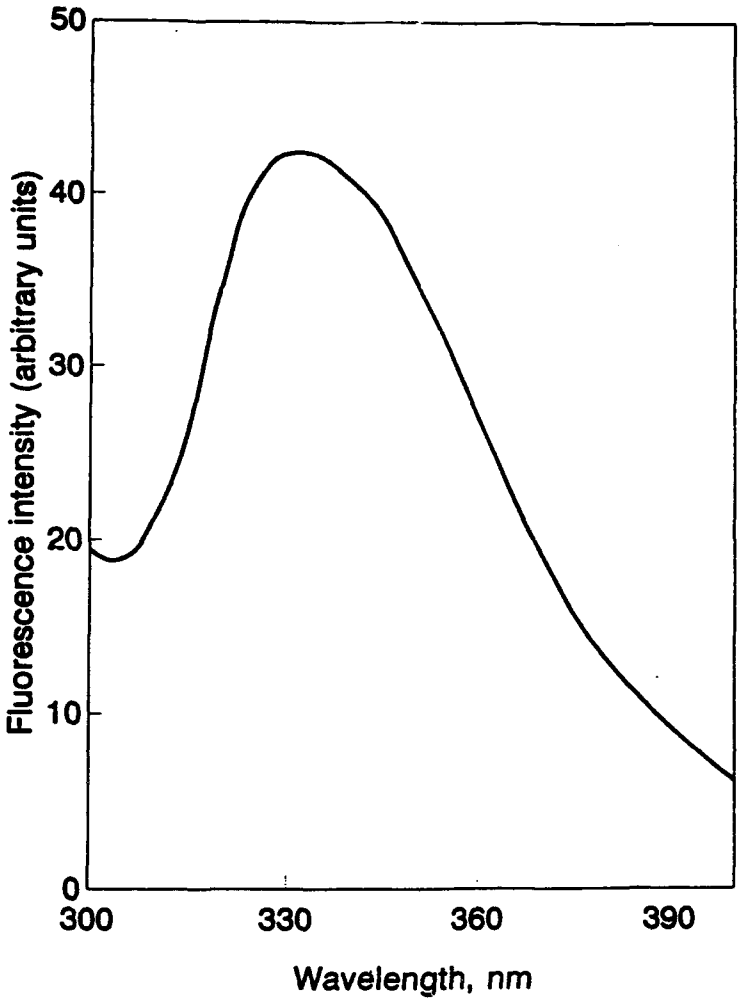



*Figure 25: Far UV CD spectrum of refolded and misfolded rhAChE. The spectra of the purified active rhAChE sample, containing 0.37mg/ml in PBS pH 7.4, and of the misfolded (inactive misfolded) rhAChE sample at a concentration of 1.32mg/ml in the same buffer, were obtained at a scanning speed of four readings per nm and averaged to give the final spectrum. Protein concentrations were derived from $\varepsilon_{1\%}$ = 23.1 for the active enzyme and $\varepsilon_{1\%}$ = 16.2 for the misfolded protein both at 280nm.*
*Active purified rhAChE – ■–■, misfolded inactive rhAChE, ●–●.*




hg-mo-02

*Figure 26: Fluorescence emission spectrum of purified active rhAChE. The emission spectrum was determined on a sample with a protein concentration of 20µg/ml in PBS, pH 7.4, with a Jasco spectrofluorometer Model FP770 at a temperature of 25°C. Excitation wavelength was 295nm with excitation and emission slits set at 5nm.*

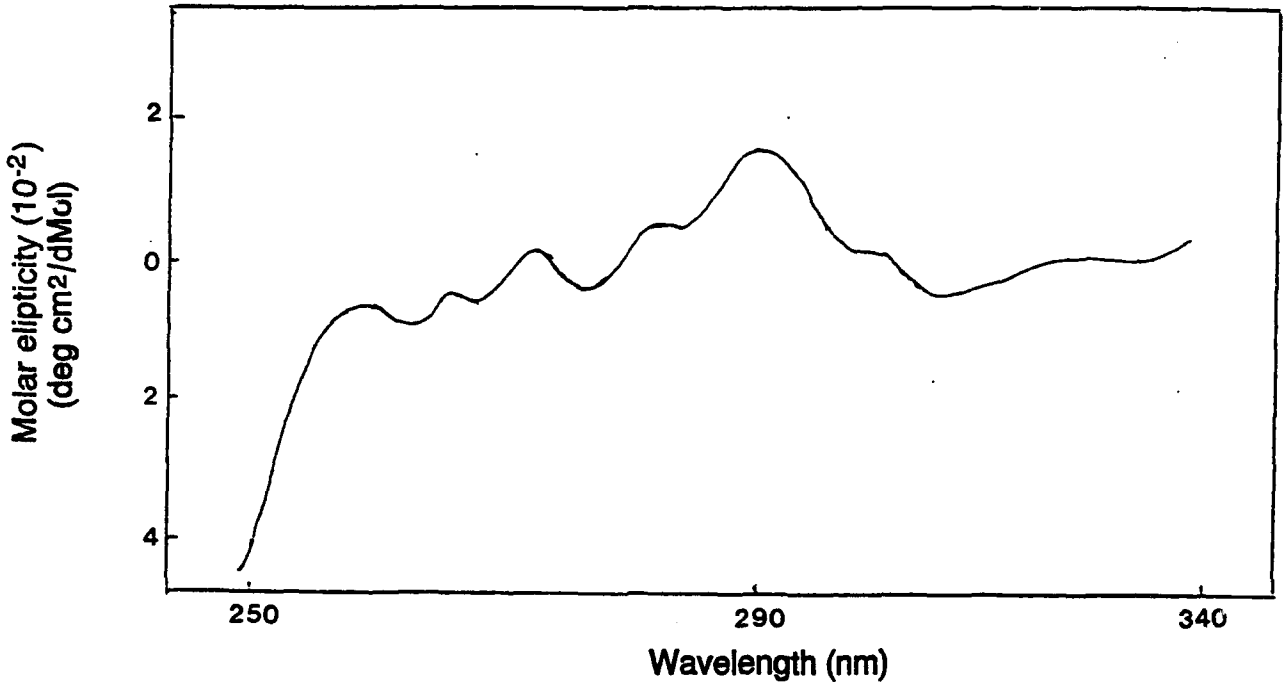



*Figure 27: Near UV CD spectrum of refolded AChE. The sample (No. 3) in PBS pH 7.4 had a concentration of 0.37mg/ml, based on the average $\varepsilon_{1\%}$ value of 22.2 at 280nm (Table 10). The spectrum was obtained on a Jasco spectropolarimeter model 500, at a scanning speed that ensured at least four readings per nm of scan. Four scans were averaged to give the final spectrum. A 1cm cylindrical cuvette was used.*

3.4.12 Peptide mapping

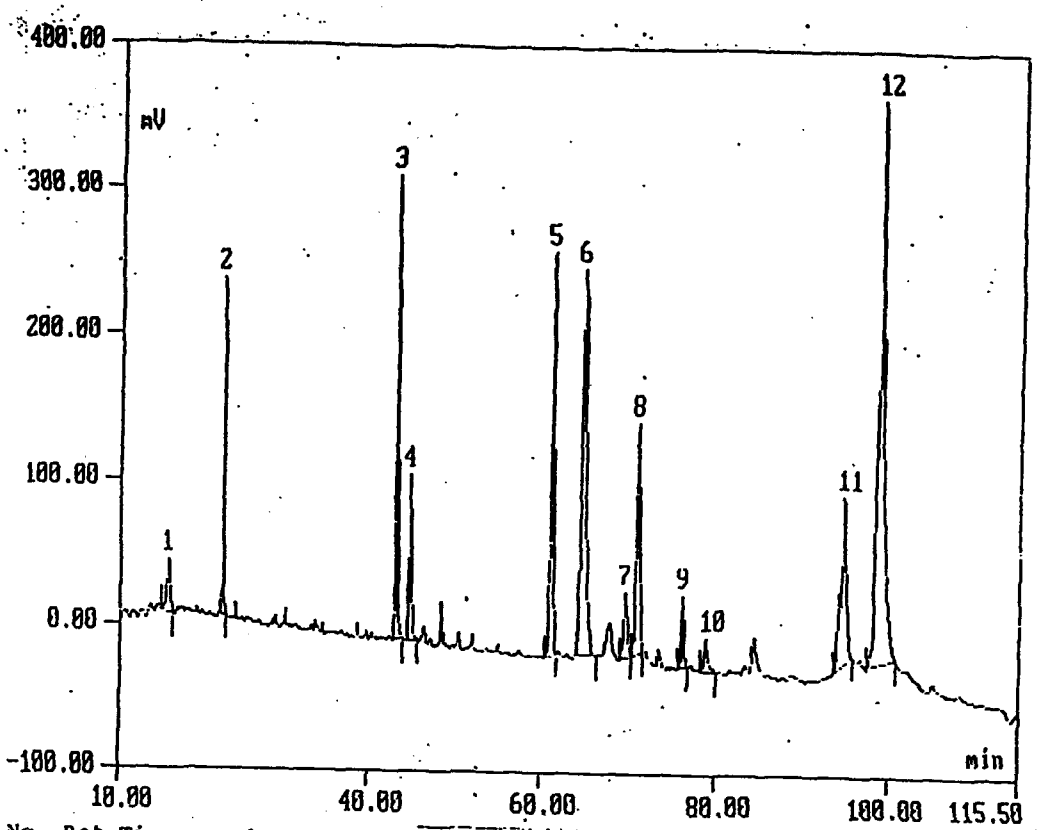
3.4.12.1 rhAChE fingerprint: The cleavage product of rhAChE treated with endoproteinase Lys-C subjected to HPLC analysis under the conditions described in the Experimental Procedures Section is shown in Figure 28. Four independent runs listing the retention time and the relative area (percentage) are shown for each peak in Tables 12 and 13, respectively. A relative era of less than 1% of the total was programmed not to be registered. Fraction #10 in run #2 fell short of 1% and therefore is not listed. In general, Fraction #10 is only slightly above 1% and may be regarded as a "false positive". The true identity of Fraction #10 was revealed after amino acid sequencing, which indicated that no peptide was present (Table 14).

The retention times of each of the eluted peptides are similar with minor experimental fluctuations and are considered reproducible. Hence, the HPLC fingerprint obtained for rhAChE with endoproteinase Lys-C coincides with the expected number of peptides predicted from the amino acid sequence.

The identity of the various peaks in the RP-HPLC elution profile of the Lys-C digest of rhAChE (Figure 28) was determined by N-terminal sequencing of the peptides. From each fraction obtained from the above elution profile, >50% (~4ml) was evaporated to dryness in a Speed-Vac concentrator (Savant Instr., Model RH 24-18, Farmingdale, NY).

3.4.12.2 Determination of the N-terminal amino acid sequence: The dried material was re-dissolved in 200µl of 0.1% TFA in acetonitrile, and the solutions were transferred to 1.5ml Eppendorf tubes where they were again evaporated to dryness as before. The samples were then dissolved in 50µl 0.1% trifluoroacetic acid and applied on the filter disc. Five Edman degradation cycles (Hunkapillar and Hood, 1978; Hunkapillar and Hood, 1983) were performed. Sequence analyses were performed at the Weizmann Institute of Science, Rehovot, Israel, on a gas phase protein micro-sequencer, model 475A (Applied Biosystems), using the sequencing program of Applied Biosystems. The resulting PTH-amino acids are analyzed at 269nm by a High Performance Liquid Chromatography (HPLC) System, model 120A (Applied Biosystems) - 10% of the total PTH-amino acids were usually applied on the HPLC column. 60 pmol of PTH-amino acid standards (Pierce) were used for comparison. Using myoglobin as standard, the repetitive yields of the sequencer were in the range of 95-97%. The results are presented in Table 14.

In conclusion, it can be stated that nine out of the eleven expected peptides were identified. Peak #3 in the profile contained almost equal amounts of peptides #8 and #9 in the sequence. On the other hand, peaks #7 and #8 in the profile revealed the same amino acid sequence corresponding to peptide #7. Peaks #10 and #11 yielded no detectable sequence, and peptides #3 (from aa #53 to #332) and #4 (from aa #332 to #348) were not identified in the profile.




| No. | Ret.Time min | Area mV*min | Rel.Ar % |
|---|---|---|---|
| 1 | 15.82 | 18.26 | 1.93 |
| 2 | 22.40 | 41.55 | 4.40 |
| 3 | 43.03 | 88.01 | 9.32 |
| 4 | 44.61 | 34.79 | 3.69 |
| 5 | 60.98 | 101.85 | 10.79 |
| 6 | 64.45 | 160.50 | 17.00 |
| 7 | 69.36 | 22.54 | 2.39 |
| 8 | 70.74 | 78.58 | 8.32 |
| 9 | 76.01 | 17.09 | 1.81 |
| 10 | 78.78 | 12.98 | 1.38 |
| 11 | 94.59 | 82.29 | 8.72 |
| 12 | 98.73 | 285.51 | 30.25 |

*Figure 28: HPLC fingerprint. 450µg of rhAChE was digested with endoproteinase Lys-C as described in the Experimental Procedures Section. 100µl of the digest was injected for analysis.*

TABLE 12

rhAChE FINGERPRINT – RETENTION TIME (min)

| PEAK | I | II | III | IV | AVG | S.D. | C.V.% |
|---|---|---|---|---|---|---|---|
| 1 | 17.31 | 16.56 | 15.88 | 15.82 | 16.39 | 0.60 | 3.69 |
| 2 | 23.63 | 22.96 | 22.43 | 22.40 | 22.86 | 0.50 | 2.19 |
| 3 | 45.13 | 43.91 | 43.12 | 43.03 | 43.80 | 0.84 | 1.92 |
| 4 | 46.41 | 45.32 | 44.66 | 44.61 | 45.25 | 0.73 | 1.60 |
| 5 | 63.16 | 62.00 | 60.93 | 60.98 | 61.77 | 0.91 | 1.47 |
| 6 | 65.81 | 64.82 | 64.40 | 64.45 | 64.87 | 0.57 | 0.87 |
| 7 | 71.13 | 70.09 | 69.14 | 69.36 | 69.93 | 0.78 | 1.11 |
| 8 | 72.57 | 71.51 | 70.52 | 70.74 | 71.34 | 0.80 | 1.12 |
| 9 | 77.70 | 76.71 | 75.73 | 76.01 | 76.54 | 0.76 | 0.94 |
| 10 | 80.20 | – | 78.51 | 78.78 | 79.16 | 0.74 | 0.94 |
| 11 | 95.15 | 95.17 | 95.55 | 94.59 | 92.12 | 0.34 | 0.36 |
| 12 | 99.16 | 99.20 | 99.78 | 98.73 | 99.22 | 0.37 | 0.38 |

Roman numerals indicate Run 1 through Run 4

AVG = average
S.D.= standard deviation
C.V.% = coefficient of variance

TABLE 13

rhAChE FINGERPRINT - RELATIVE PEAK AREA

| PEAK | I | II | III | IV | AVG | S.D. | C.V.% |
|---|---|---|---|---|---|---|---|
| 1 | 1.54 | 1.36 | 1.65 | 1.89 | 1.61 | 0.19 | 11.92 |
| 2 | 4.24 | 4.10 | 3.94 | 4.31 | 4.15 | 0.14 | 3.42 |
| 3 | 9.39 | 9.67 | 8.47 | 9.12 | 9.16 | 0.44 | 4.85 |
| 4 | 3.28 | 3.00 | 3.39 | 3.61 | 3.32 | 0.22 | 6.62 |
| 5 | 11.20 | 10.01 | 10.68 | 10.56 | 10.61 | 0.42 | 3.99 |
| 6 | 16.61 | 15.79 | 15.95 | 16.65 | 16.25 | 0.38 | 2.37 |
| 7 | 4.41 | 3.53 | 4.04 | 2.34 | 3.58 | 0.78 | 21.82 |
| 8 | 6.94 | 7.91 | 6.68 | 8.15 | 7.42 | 0.62 | 8.39 |
| 9 | 1.83 | 1.90 | 1.74 | 1.77 | 1.81 | 0.06 | 3.38 |
| 10 | 1.40 | - | 1.28 | 1.35 | 1.34 | 0.05 | 3.66 |
| 11 | 8.84 | 8.79 | 9.39 | 9.38 | 9.10 | 0.29 | 3.14 |
| 12 | 30.32 | 33.93 | 32.78 | 30.87 | 31.98 | 1.45 | 4.54 |

Roman numerals indicate Run 1 through Run 4

AVG = average
S.D.= standard deviation
C.V.% = coefficient of variance

TABLE 14

ASSIGNMENT OF THE PEAKS IN THE RP-HPLC ELUTION PROFILE OF THE Lys-C DIGEST TO PEPTIDES IN THE AChE SEQUENCE

| PEAK # | RT (min) | PEPTIDE POSITION IN SEQUENCE # | N-TERMINAL SEQUENCE FOUND | PEPTIDE SIZE (#aa) | LOCATION IN SEQUENCE (#-#) | AMOUNT SEEN IN SEQUENCE (pmol) |
|---|---|---|---|---|---|---|
| 1 | 15.82 | 11 | QDR?S | 7 | 577-583 | 400-700 |
| 2 | 22.40 | 10 | NQFDH | 8 | 569-576 | 700-1000 |
| 3 | 43.03 | 8 | LLSAT | 16 | 539-554 | 500-700 |
| | | 9 | AEFHR | 14 | 555-568 | 500-580 |
| 4 | 44.61 | 1 | MEGRE | 24 | Met+ 1-23 | 400-630 |
| 5 | 60.98 | 6 | IFAQR | 26 | 471-496 | 640-680 |
| 6 | 64.45 | 2 | TPGGP | 30 | 24-53 | 160-350 |
| 7 | 69.36 | 7 | APQ?P | 42 | 497-538 | 10-14 |
| 8 | 70.74 | 7 | APQ?P | 42 | 497-538 | 77-85 |
| 9 | 76.01 | 8 | LLSAT | 16 | 539-554 | 7-13 |
| 10 | 78.78 | - | - | - | - | - |
| 11 | 94.59 | - | - | - | - | - |
| 12 | 98.73 | 5 | DNESL | 122 | 349-470 | 10-12 |

*NOTE: Peak # refers to HPLC elution peaks; RT - retention time; peptide position in sequence - location of the peptide relative to the N-terminus starting at EGRE...*

# 4. DISCUSSION

## 4.1 rhAChE expression, reconstitution and purification

The DNA sequence encoding the human AChE catalytic subunit was inserted into *E. coli* expression vectors under control of $\lambda P_L$ or the *E. coli* deo promoters. Cultures harboring these vectors failed to produce the cloned gene product as determined by SDS-PAGE or Western blot analysis. The lack of rhAChE expression in clones A4255pAIF-4 and Sϕ930pAIF-11 is attributed to the high GC content of the cloned gene flanking the ribosomal binding site. GC-rich sequences in mRNA in the vicinity of the ribosomal binding site often generate stem-loop structures which may block translation (Kozak, 1983; Looman et al., 1986). Disruption of the GC-rich sequences at the 5'-end of the cloned hAChE indeed resulted in high expression levels of rhAChE in clones A4255pAIF-34 and Sϕ930pAIF-52, driven by $\lambda P_L$ and deo promoters, respectively.

The rhAChE expressed in *E. coli* accumulates as an aggregate in inclusion bodies, as observed for a number of other cloned eukaryotic genes in *E. coli* (Mitraki and King, 1989), and possesses no AChE activity. Solubilization and refolding of the 62kD polypeptide into an enzymatically active form was achieved in the presence of L-arginine and GSSG, which were shown to be effective in refolding of the Fab fragment produced in *E. coli* (Buchner and Rudolph, 1991). The attainable enzyme activities reconstituted *in vitro* and derived from clones Sϕ930pAIF-52 were low (Table 2). We suspected that the odd number of seven cysteine residues in the hAChE (Soreq et al., 1990) enhanced incorrect disulfide bond formation during refolding. Indeed, replacement of cysteine 580 by serine resulted in a considerably higher yield of active rhAChE upon refolding (Table 2). The specific activity of reconstituted enzyme prepared from inclusion bodies produced in Sϕ930pMFL-52Ser ranged between 20 and 46 U/mg protein.

The data obtained from the gel permeation chromatography of highly purified rhAChE reveal that the active enzyme is a monomer. Velan et al. (1991b) have recently shown that replacement of $Cys^{580}$ with Ala resulted in secretion of predominantly monomeric hAChE from transiently transfected human embryonic kidney cell line 293. The active rhAChE monomer produced in tissue culture differs from rhAChE from *E. coli* in that the latter enzyme is not glycosylated since *E. coli* does not harbor a glycosylating system. Hence, glycosylation appears not to be essential for catalytic activity.

The rhAChE catalytic subunit containing the $Cys^{580} \rightarrow Ser^{580}$ substitution, expressed in *E. coli* as aggregated inactive enzyme, was solubilized by a strong denaturant and refolded *in vitro* by a procedure developed for large-scale operation. The refolded rhAChE was purified to apparent homogeneity and revealed a single protein band on SDS-PAGE stained with Coomassie Brilliant Blue (Figure 14). The specific activity (S.A.) obtained ranged from 4572 to 5936 U/mg protein (Tables 7-9) and is essentially similar to the S.A. reported for the AChE purified from erythrocytes (Rosenberry and Scoggin, 1984).

## 4.2 Catalytic properties

The catalytic properties of the reconstituted rhAChE, as demonstrated by substrate specificity and selective inhibition by BW284C51 and iso-OMPA, are quite similar to those of native AChE derived from erythrocytes (Figures 16, 18) and the rhAChE expressed in tissue culture (Velan et al., 1991a; 1991b). The *E. coli*-derived enzyme and the erythrocyte AChE show a 50% inhibition at 1nM BW284C51 while the enzyme produced in the eukaryotic expression system was inhibited at 8nM (Velan et al., 1991a). The 50% inhibitory concentration of iso-OMPA is in the 0.8 - 1mM range for the native and the recombinant AChE and further elucidates the "true" nature of the *E. coli*-derived enzyme.

## 4.3 rhAChE stability

Purified human erythrocyte AChE diluted to low protein concentration (7.5µg/ml) was shown to be unstable in the absence of protein carrier (Ciliv and Ozand, 1972). However, activity was fully retained in the presence of BSA. The *E. coli*-derived rhAChE was unstable at low protein concentrations of 0.6-1µg/ml with a 50% inactivation observed within 60 min at 22°C. Similar to the observations reported for the human erythrocyte enzyme, rhAChE was stabilized by 0.5mg/ml BSA as carrier.

The pH stability profiles of human erythrocyte AChE and rhAChE presented in Figure 20 show that the recombinant enzyme is less stable in the pH range of 4.0-8.0. The reduced stability of the rhAChE could be attributed to the difference in structure between the two enzymes. While the active rhAChE was shown to be monomeric (Figure 15), a disulfide-linked dimer structure characterizes the human erythrocyte AChE (Rosenberry and Scoggin, 1984). We cannot rule out, however, the possibility that the observed difference originates from additives present in the commercial preparation of the human erythrocyte AChE.

## 4.4 Primary structure and pI

The pI of the rhAChE could not be determined precisely in view of the diffuse band obtained after electrophoresis. It is clear from Figure 23 that the pI of the rhAChE is within the range of 5.5-5.8 and this suggests that some degree of aggregation may have contributed to the diffuse appearance of the rhAChE band. The pI of purified human erythrocyte AChE determined by electrophoresis in sucrose gradient was between 4.55 and 5.18 (Ott et al., 1975) and contained five isoforms.

The comparison between rhAChE and the human erythrocyte AChE is based on the mounting evidence that in humans a single gene encodes the entire repertoire of acetylcholinesterases (Li et al., 1991; Erlich et al., 1992; Getman et al., 1992). The deduced amino acid sequence (Soreq et al., 1990) of the mature catalytic AChE subunit was predicted to be identical up to amino acid 544 as illustrated by Ben Aziz-Aloya et al. (1993). Indeed, the amino acid sequencing data of 5 tryptic peptides confirmed the identity of 94 of the 97 amino acids (Chhajlani et al., 1989).

However, the amino acid sequence at the C-terminus may differ considerably. A stretch of 13 and 40 amino acids extending downstream from $Tyr^{544}$ differentiates between the ampiphilic and hydrophilic forms, respectively (Roberts et al., 1991; Soreq et al., 1990; Li et al., 1991). The rhAChE expressed in *E. coli* contains the 40-amino-acid-long C-terminus, while the human erythrocyte AChE possesses the shorter 13 amino acid long sequence. Considering that the amino acid composition of rhAChE (Table 11) differs from that of human erythrocyte AChE (Rosenberry and Scoggin, 1984), it is expected that these differences will be reflected in the pI values of the two proteins.

## 4.5 Absorption coefficient

The absorption coefficient at 280 nm is based on the determination of protein concentration obtained by two independent methods: i) the amino acid analysis data; ii) the modified Bradford method (Macart & Gerbaut, 1982). The corresponding $\varepsilon_{1\%}$ values for rhAChE, 23.1 and 22.2, are in excellent agreement. On the other hand, the $\varepsilon_{1\%}$ - at 280nm - of *Electrophorus* AChE reported by Rosenberry et al. (1972) varied considerably and ranged between 17.2±0.3 and 21.8±0.7, depending on the method of determination used. The relatively high $\varepsilon_{1\%}$ value of 23.1, i.e., $\varepsilon_M$=149.5x$10^3$ $M^{-1}$ $cm^{-1}$ obtained for the refolded active rhAChE, is typical of a Trp-rich protein. Indeed, from the deduced amino acid sequence (Soreq et al., 1990) it can be calculated that rhAChE contains 16 Trp and 21 Tyr residues. The Trp contribution to the absorption spectrum is predominant, since the molar absorbencies of Trp and Tyr are 5600 $M^{-1}$ $cm^{-1}$ at 279nm and 1420 $M^{-1}$ $cm^{-1}$ at 275nm (Fasman, 1976), respectively. The molar absorbencies of Trp, Tyr and cystine residues (110 $M^{-1}$ $cm^{-1}$ at 280nm), as well as the number of these residues in the amino acid sequence, were used to calculate the theoretical minimum $\varepsilon_M$ of 119.8x$10^3$ $M^{-1}$ $cm^{-1}$, i.e., $\varepsilon_{1\%}$=18.5 at 280nm. While the values of $\varepsilon_{1\%}$ of rhAChE resemble those reported for the *Electrophorus* AChE, the $\varepsilon_{1\%}$ values - at 280nm - reported for fetal bovine AChE were much lower and ranged between 10.4 and 11.9 (Ralston et al., 1985).

## 4.6 Secondary structure and environment of aromatic residues

The secondary structure obtained by far UV CD for the refolded active rhAChE molecule (39% α-helix and 22% ß-sheet) agrees well with the published information on a globular acetylcholinesterase from another source (*Torpedo californica*), although in this case tetrameric or dimeric forms of the enzyme are described (Manavalan et al., 1985; Sussman et al., 1991). The far UV CD for the *Torpedo* AChE is compatible with an α/ß structure made up of 33% helical structure and 23% of both parallel and antiparallel ß-sheets (Manavalan et al., 1985). The CD values for the *Torpedo* AChE were recently found to be in reasonable agreement with those obtained in the three-dimensional structure determined by X-ray analysis to 2.8A resolution (Sussman et al., 1991): 30% α-helix and 15% ß-sheet. On the other hand, our far UV CD data for the misfolded rhAChE molecule show that it has an inverted content of α-helix and ß-sheet: it consists of only 24% of α-helix and has almost a double content (42%) of ß-sheet. Thus, it seems that some misfolding into ß-sheet occurs during refolding and the molecule probably remains trapped in this altered conformation.

Intrinsic fluorescence is a structural method which can be used to monitor the environment of the aromatic residues in proteins. Thus, compared to the λmax of the emission spectrum of the Trp model compound N-acetyl-tryptophanamide, the 20 nm blue-shifted spectrum of AChE is indicative of an average apolar environment for its Trp residues (Van Duuren, 1961). Furthermore, the fluorescence efficiency, $R_{Trp}$ (Cowgill, 1968) of rhAchE - compared to that of the model compound - was found to be 1.17. This also indicates that, on the average, the Trp residues of AChE are less quenched than the model compound, which is also in agreement with the above assumption of an average more apolar environment for its Trp residues.

## 4.7 Peptide mapping

Lys-C digestion of rhAChE purified from *E. coli* Sϕ930pMFL-52Ser generated 12 peaks after HPLC analysis. Only nine of the expected 11 peaks matched the predicted peptide fragments to be obtained by the cleavage. The two peptides that were not recovered correspond to peptides 281 amino acids and 16 amino acids in length, which represent peptides numbers 3 and 4 in the AChE sequence (Soreq, 1990), respectively. It is surprising that peptide number 8 eluted in two different peaks and may indicate that this peptide interacts (or aggregates) with peptide number 9, as they co-eluted also in peak 3 (see Table 14). We have attempted to generate a peptide map using trypsin; however, we have encountered severe solubility problems with the peptides generated. It is possible that the two missing peptides did not dissolve after evaporation, and therefore no amino acid sequences were recovered from peaks 10 and 11.

## 4.8 Amino acid substitutions and deletions that affect enzyme function

A number of amino acid substitutions and deletions within the AChE amino acid sequence, generated by manipulating its DNA sequence, had adversely affected the activity of the enzyme. Replacement of $Cys^{580}$ by Ser in plasmid pMFL-52Ser and deletion of 43 amino acids of the C-terminus of AChE (plasmid pMFEG-8) resulted in the recovery of a functional enzyme after refolding *in vitro* (Tables 2 and 3). In both cases, the catalytic properties of the enzyme were very similar, as determined by substrate specificity (Figures 10 and 16) with acetylthiocholine. On the other hand, substitution of $Cys^{257}$ and $Cys^{272}$ by Ser (pMFE-5234) yielded very poor enzyme activity after refolding. The specific activity obtained was 100-fold lower compared to S.A. obtained with the nonmutated enzyme (pAIF-52).

Based on results obtained with the $Cys^{580}$ substitution to $Ser^{580}$, which enhanced yields of enzymatically active AChE, we expected that replacement of cysteine residues 257 and 275 by serine may further enhance recovery of active enzyme. The substantial reduction in enzyme activity of AChE derived from Sϕ930pMFE-5234 could be explained on the assumption that the three dimensional structure of rhAChE subunit is similar to that of the *Torpedo* enzyme (Sussman et al., 1991). The two cysteine residues in *Torpedo* were shown to reside on the surface of the subunit and link two ß-structures in the subunit by disulfide linkage. If indeed this is the case in the rhAChE subunit, then replacement of cysteine residues 257 and 272 will eliminate the formation of the disulfide bridge holding the two ß-structures together. Consequently, the tertiary structure of the subunit is predicted to change and therefore impair enzymatic activity. In conclusion, the data indicate that the two cysteine residues at positions 257 and 272 are important for the structural integrity of the rhAChE subunit.

The rhAChE derived from Sϕ930pMFEG-89 that harbors deletions at both C- and N-termini yielded no active enzyme after refolding. Since the parental clone that harbors the 43-amino-acid C-terminus (pMFEG-8) yielded enzymatically active AChE after reconstitution, the lack of activity implies that the first 30 amino acids at the N-terminus harbor residues that were crucial for obtaining the correct structure of the protein required to be functional.

## TASK AND ACHIEVEMENTS

Contractor tasks have been defined in USAMRDC Log No. 88351002 dated 1990 June 22. Table 15 below summarizes the achievements by tasks.

### TABLE 15

### ACHIEVEMENT SUMMARY

| Tasks | | Status |
|---|---|---|
| 1. | Preparation of full-length cDNA clone | Full-length cDNA was provided by Prof. H. Soreq |
| 2. | Construction of AChE expression vectors<br>2.1 Thermoinducible ($\lambda P_L$ driven)<br>2.2 Constitutive (deo promoter driven) | Completed |
| 3. | Optimization of AChE expression and scale-up fermentation<br>3.1 $\lambda P_L$ expression system<br>3.2 Constitutive expression system | Completed |
| 4. | Development of an AChE purification scheme<br>4.1 Assessment of biological activity<br>4.2 Reconstitution of inactive AChE to enzymatically active form<br>4.3 Purification process to obtain highly purified rhAChE | <br>Completed<br>Completed<br>Completed |
| 5. | Characterization of recombinant AChE<br>5.1 M.W. determination of rhAChE<br>5.2 Effect of reducing agent<br>5.3 Western analysis<br>5.4 Enzymatic activity in solution and nondenaturing gels<br>5.5 Substrate and inhibitor specificity<br>5.6 Final recombinant AChE product characterization<br>5.6.1 Isoelectric focusing<br>5.6.2 Two dimensional<br>5.6.3 Peptide mapping<br>5.6.4 Amino acid analysis<br>5.6.5 Amino acid sequencing<br>5.6.6 U.V. absorption spectroscopy<br>5.6.7 Fluorescence emission spectroscopy<br>5.6.8 Circular dichroism<br>5.6.9 Extinction coefficient<br>5.6.10 Stability | <br>Completed<br>Completed<br>Completed<br>Completed<br>Completed<br><br>Completed<br>Not determined<br>Completed<br>Completed<br>Partial sequencing<br>Completed<br>Completed<br>Completed<br>Completed<br>Completed |
| 6. | Scale-up purification process and production of rhAChE | Completed |

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